REMARKS

In the Office Action dated April 22, 2005, claims 49, 54-59, 64-69, 74-79 and 84-88 were examined with the result that all claims were rejected. The Examiner made the rejection final. In response, Applicant has filed a Request for Continuing Examination (RCE) together with the following remarks. Reconsideration of this application is requested.

In the Office Action, claims 49 and 54-58 were provisionally rejected under the Doctrine of Obviousness Type Double Patenting as being unpatentable over claims 12-22 of copending Application No. 10/669,990. Although the conflicting claims are not identical, the Examiner indicated that the compound in the co-pending application '990 and the compound defined by claim 49 in the present application '103 are homologs of each other since the presently claimed compounds have one additional methyl group at the 26 and 27 carbon positions of the side chain. In addition, the claims in application '990 also are directed toward various cancerous diseases. Therefore, the Examiner concludes that since homologs are known to have similar properties, the presently claimed methods would have been obvious to one of ordinary skill in the art. Applicant, however, disagrees with the Examiner's conclusion for the following reasons.

The biological activities of the presently claimed 26,27-dihomo compound can be found in the specification as filed in Tables 3 and 4 on page 28 thereof. First, with regard to the calcemic activity data in Table 4, these data indicate that the 26,27-dihomo compound claimed herein is significantly more active than 1α,25-dihydroxyvitamin D_3 in both intestinal calcium transport as well as bone calcium mobilization (serum calcium). This is evidenced by the fact that at a dosage of 32 pmol, the claimed 26,27-dihomo compound has calcium transport and serum calcium values higher than 1α,25-dihydroxyvitamin D_3 which was administered at a dosage of 260 pmols. Thus, even though 1α,25-dihydroxyvitamin D_3 was administered at almost 10 times the dose of the 26,27-dihomo compound, the natural hormone still had calcium transport and serum calcium values that were less than the claimed 26,27-dihomo compound. Therefore, it

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can be stated that the 26,27-dihomo compound claimed herein has significant and very potent calcemic activity that is greater than 1α,25-dihydroxyvitamin D_3.

With regard to cell differentiation activity, Applicant refers the Examiner to Table 3 on page 28 of the present patent application wherein the cell differentiation activity and vitamin D receptor (VDR) binding data is presented. Table 3 illustrates that although the 26,27-dihomo compound claimed herein has less binding activity than 1α,25-dihydroxyvitamin D_3, it is more active in HL-60 cell differentiation activity. This latter activity is evidenced by the fact that a significantly lower concentration of the 26,27-dihomo compound is needed to obtain the ED_{50} value. Applicant refers the Examiner to the discussion at page 28, lines 15-27. As a result of these data, it can be concluded that due to its significantly greater cell differentiation activity, a lower dosage of the 26,27-dihomo compound is necessary for obtaining the same activity as 1α,25-dihydroxyvitamin D_3.

The following chart summarizes the above biological activities for the 26,27-dihomo compound of claim 49:

Compound	Intestinal Ca Transport	Bone Ca Mobilization (Serum Ca)	Cell Differentiation
20(S)-1α,25-dihydroxy-2-methylene-26,27-dihomo-19-norvitamin D_3	significantly greater* (Table 4)	significantly greater* (Table 4)	Significantly greater* (Table 3)

*As compared to the natural hormone 1α,25-dihydroxyvitamin D_3.

The homolog disclosed in the '990 application is 2-methylene-19-nor-20(S)-1α,25-dihydroxyvitamin D_3 (sometimes referred to as 2MD). The calcemic activity for compound 2MD is set forth in Table 1 on page 24 of the present patent application '103. Also, the VDR binding activity for 2MD is illustrated in Figure 1 while the cell differentiation activity of 2MD is illustrated in Figure 2 of the present '103 application. In addition, the calcemic and cell differentiation activity of 2MD is also set forth in U.S.

Patent 5,843,928. The '928 patent summarizes the calcemic activity of 2MD beginning at column 15, line 63 and continuing through column 16, line 11 as follows:

> "Surprisingly, however, the 2-methylene substitutions produced highly selective analogs with their primary action on bone. When given for 7 days in a chronic mode, the most potent compound tested was the 2-methylene-19-nor-20S-1,25-$(OH)_2D_3$ (Table 1). When given at 130 pmol/day, its activity on bone calcium mobilization (serum calcium) was of the order of at least 10 and possible 100-1,000 times more than that of the native hormone. Under identical conditions, twice the dose of 1,25-$(OH)_2D_3$ gave a serum calcium value of 13.8 mg/100 ml of serum calcium at the 130 pmol dose. When give at 260 pmol/day, it produced the astounding value of 14 mg/100 ml of serum calcium at the expense of bone. To show its selectivity, this compound produced no significant change in intestinal calcium transport at either the 130 or 260 pmol dose, while 1,25-$(OH)_2D_3$ produced the expected elevation of intestinal calcium transport at the only dose tested, i.e. 260 pmol/day.
>
> ***
>
> These results illustrate that ...the 20S-2-methylene derivatives of 19-nor-1,25-$(OH)_2D_3$ are selective for the mobilization of calcium from bone."

Thus, it can be concluded from the above quotation and the data contained in Table 1 at page 24 of the present patent application '103 that the 2MD compound has little, if any, intestinal calcium transport activity but very potent bone calcium mobilization activity at the doses tested.

With regard to competitive binding activity, the 2MD analog "bound equally well to the porcine receptor (as the natural hormone 1α,25-dihydroxyvitamin D_3). See Figure 1 and the description at page 23, lines 1-2 of the present patent application '103.

With regard to cell differentiation activity, Figure 2 of the present patent application '103 illustrates that the analog 2MD is more potent in inducing differentiation of HL-60 cells to the monocyte, as compared to 1α,25-dihydroxyvitamin D_3. This is clearly evidenced by the fact that at the same concentration, a higher percent

differentiation value is obtained for the 2MD analog as compared to 1α,25-dihydroxyvitamin D_3. Thus, it can be concluded that the analog 2MD is more active than 1α,25-dihydroxyvitamin D_3 in inducing differentiation of HL-60 cells to the monocyte.

The following chart summarizes and compares the biological activities of the analog 2MD with the presently claimed 26,27-dihomo compound:

Compound	Intestinal Ca Transport	Bone Ca Mobilization (Serum Ca)	Cell Differentiation
20(S)-1α,25-dihydroxy-2-methylene-26,27-dihomo-19-norvitamin D_3	significantly greater* (Table 4)	significantly greater* (Table 4)	Significantly greater* (Table 3)
20(S)-2-methylene-19-nor-1α,25-dihydroxyvitamin D_3 (2MD)	"no significant change"* (page 23, lines 11-14) (Table 1)	"at least 10 and possibly 100-1000 times" more active* (page 23, lines 6-11) (Table 1)	"extremely potent"* (page 23, lines 21-22) (Fig. 2)

*As compared to the natural hormone 1α,25-dihydroxyvitamin D_3.

The comparison in the above chart demonstrates that the analog 2MD has little, if any, intestinal calcium transport activity, extremely high bone calcium mobilization activity, and extremely high cell differentiation activity, when compared to 1α,25-dihydroxyvitamin D_3. In contrast, the presently claimed 26,27-dihomo compound has intestinal calcium transport activity that is significantly greater than 1α,25-dihydroxyvitamin D_3, bone calcium mobilization activity that is significantly greater than 1α,25-dihydroxyvitamin D_3, and cell differentiation activity that is also higher than 1α,25-dihydroxyvitamin D_3. Thus, the presently claimed 26,27-dihomo compound has significantly different calcium transport activity than the analog 2MD. Clearly, such activities would not be predicted based upon the structural similarity of the two compounds. One skilled in the art would have predicted that the compounds should have

approximately the same intestinal calcium transport activity due to their structural similarity, but instead, it is clear that the presently claimed 26,27-dihomo compound has much higher intestinal calcium transport activity than the analog 2MD. As a result, Applicant believes these properties are unexpected and provide a basis for unobviousness over the 2MD analog.

Accordingly, Applicant believes the Examiner should withdraw the obviousness type double patent rejection based on the analog 2MD.

In the Office Action, the Examiner also rejected claims 49, 54-59, 64-69, 74-79 and 84-88 under 35 USC §112, first paragraph, because the Examiner alleges that the specification is not enabling for the method of use of all the claims. The Examiner states that the claims are too broad in that they are drawn to a method of treating too many cancerous diseases, and believes there is not enough guidance presented in the disclosure on how to use the invention successfully for the treatments of "so many cancerous diseases" with the result that undue experimentation would be required of one skilled in the art. Applicant, however, disagrees for the following reasons.

First of all, the Examiner states that the claims encompass "cancerous diseases, including leukemia, colon cancer, breast cancer, prostate cancer, etc." and are thus too broad. However, Applicant would like to point out that in the Amendment dated January 5, 2005, Applicant amended independent claims 49, 59, 69 and 79 to limit the term "cancerous disease" by inserting a Markush grouping defining the cancerous diseases as leukemia, colon cancer, breast cancer, or prostate cancer. Thus, each of the independent claims are now limited to only four types of cancers. Thus, the independent claims do not purport to encompass all cancerous diseases. The amendment referred to above clearly limits the independent claims to leukemia, colon cancer, breast cancer or prostate cancer.

There is a clear relationship between the ability of vitamin D compounds to differentiate cells and these four cancer therapies. The ability of vitamin D compounds to differentiate cells has long been used to predict efficacy against certain cancers such as leukemia, colon cancer, breast cancer and prostate cancer. Numerous patents can be cited

by Applicant in support of that proposition. In addition, Applicant includes two articles which evidence a clear relationship between vitamin D compounds, their ability to differentiate cells, and as a result, their potential use in various cancer therapies. The first article is Van Leeuwen et al, "Vitamin D: Cancer and Differentiation", Vitamin D, 2nd Edition, Volume 2, Chapter 89, pages 1571-1597 (Elsevier Academic Press, 2005). The second article is written by Jane Higdon, and is entitled "Vitamin D" and was published by the Micronutrient Information Center of The Linus Pauling Institute. It is available on the website http://lpi.oregonstate.edu/infocenter/vitamins/vitaminD. Both of these articles demonstrate that those skilled in the art recognize the cell differentiation activity of vitamin D analogs is predictive of the potential use of that analog in the treatment of leukemia, colon cancer, breast cancer and prostate cancer. As a result, Applicant believes those skilled in the art would not be required to perform "undue experimentation" in connection with the uses set forth in the independent claims.

The cell differentiation data for each of the claimed compounds is set forth in Tables 3 and 5. These data clearly evidence the potential use of these compounds against leukemia, colon cancer, breast cancer and/or prostate cancer. Thus, Applicant believes the Examiner should withdraw the §112, first paragraph rejection of the claims.

An effort has been made to place this application in condition for allowance and such action is earnestly requested.

Respectfully submitted,

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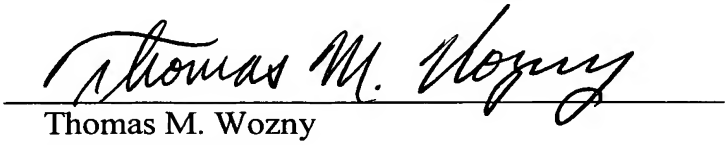
By 

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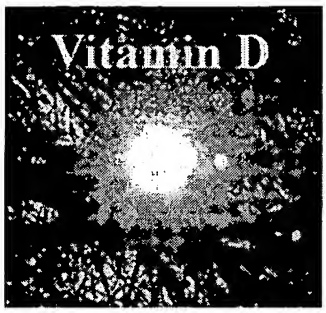

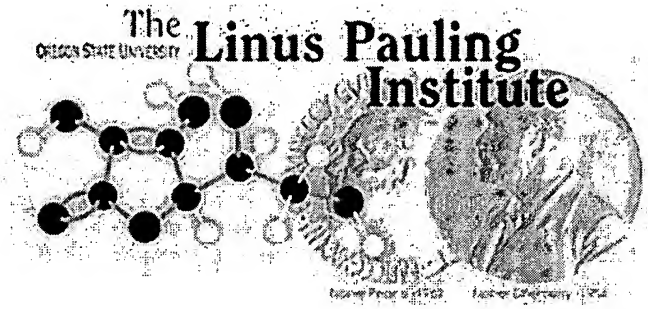

Micronutrient Information Center

VITAMIN D

Vitamin D is a fat-soluble vitamin that is essential for maintaining normal calcium metabolism (1). Vitamin D3 (cholecalciferol) can be synthesized by humans in the skin upon exposure to ultraviolet-B (UVB) radiation from sunlight, or it can be obtained from the diet. Plants synthesize vitamin D2 (ergocalciferol), which also has vitamin D activity in humans. When exposure to UVB radiation is insufficient for the synthesis of adequate amounts of vitamin D3 in the skin, adequate intake of vitamin D from the diet is essential for health.

Function

Activation of Vitamin D

Vitamin D itself is biologically inactive, and it must be metabolized to its biologically active forms. After it is consumed in the diet or synthesized in the skin, vitamin D enters the circulation and is transported to the liver. In the liver, vitamin D is hydroxylated to form 25-hydroxyvitamin D [25(OH)D], the major circulating form of vitamin D. Increased exposure to sunlight or increased intake of vitamin D increases serum levels of 25(OH)D, making the serum 25(OH)D concentration a useful indicator of vitamin D nutritional status. In the kidney and other tissues, the 25(OH)D3-1-hydroxylase enzyme catalyzes a second hydroxylation of 25(OH)D, resulting in the formation of 1alpha,25-dihydroxyvitamin D [1,25(OH)2D]—the most potent form of vitamin D. Most of the physiological effects of vitamin D in the body are related to the activity of 1,25(OH)2D (2).

Mechanisms of Action

Many of the biological effects of 1,25(OH)2D are mediated through a nuclear transcription factor known as the vitamin D receptor (VDR) (3). Upon entering the nucleus of a cell, 1,25(OH)2D associates with the VDR and promotes its association with the retinoic acid X receptor (RXR). In the presence of 1,25(OH)2D the VDR/RXR complex binds small sequences of DNA known as vitamin D response elements (VDREs), and initiates a cascade of molecular interactions that modulate the transcription of specific genes. More than 50 genes in tissues throughout the body are known to be regulated by 1,25(OH)2D (4). Some physiological responses to 1,25(OH)2D occur too rapidly to be acting through gene transcription, suggesting that their may be also be a receptor for 1,25(OH)2D on the outer membrane of cells (5).

Calcium Balance

Maintenance of serum calcium levels within a narrow range is vital for normal functioning of the nervous system, as well as for bone growth, and maintenance of bone density. Vitamin D is essential for the efficient utilization of calcium by the body (1). The parathyroid glands sense serum calcium levels, and secrete parathyroid hormone (PTH) if calcium levels drop too low (**diagram**). Elevations in PTH increase the activity of 25 (OH)D3-1-hydroxylase enzyme in the kidney, resulting in increased production of 1,25 (OH)2D. Increasing 1,25(OH)2D production results in changes in gene expression that normalize serum calcium by 1) increasing the intestinal absorption of dietary calcium, 2) increasing the reabsorption of calcium filtered by the kidneys and 3) mobilizing calcium from bone when there is insufficient dietary calcium to maintain normal serum calcium levels. Parathyroid hormone and 1,25(OH)2D are required for the latter two effects (5a).

Cell Differentiation

Cells that are dividing rapidly are said to be proliferating. Differentiation results in the specialization of cells for specific functions. In general, differentiation of cells leads to a decrease in proliferation. While cellular proliferation is essential for growth and wound healing, uncontrolled proliferation of cells with certain mutations may lead to diseases like cancer. The active form of vitamin D, 1,25(OH)2D, inhibits proliferation and stimulates the differentiation of cells (1).

Immunity

Vitamin D in the form of 1,25(OH)2D is a potent immune system modulator. The VDR is expressed by most cells of the immune system, including T cells and antigen-presenting cells, such as dendritic cells and macrophages (6). Macrophages also produce the 25(OH) D3-1-hydroxylase enzyme that converts 25(OH)D to 1,25(OH)2D (7). There is considerable scientific evidence that 1,25(OH)2D has a variety of effects on immune system function that may enhance innate immunity and inhibit the development of autoimmunity (8).

Insulin Secretion

The VDR is expressed by insulin secreting cells of the pancreas, and the results of animal studies suggest that 1,25(OH)2D plays a role in insulin secretion under conditions of increased insulin demand (9). Limited data in humans suggests that insufficient vitamin D levels may have an adverse effect on insulin secretion and glucose tolerance in type 2 diabetes (noninsulin-dependent diabetes mellitus; NIDDM) (10-12).

Blood Pressure Regulation

The renin-angiotensin system plays an important role in the regulation of blood pressure (13). Renin is an enzyme that catalyzes the cleavage (splitting) of a small peptide (Angiotensin I) from a larger protein (angiotensinogen) produced in the liver. Angiotensin converting enzyme (ACE) catalyzes the cleavage of angiotensin I to form angiotensin II, a peptide that can increase blood pressure by inducing the constriction of small arteries and increasing sodium and water retention. The rate of angiotensin II synthesis is dependent on renin (14). Recent research in mice lacking the gene encoding the VDR, indicates that 1,25(OH)2D decreases the expression of the gene encoding renin through its interaction with the VDR (15). Since inappropriate activation of the renin-angiotensin system is thought to play a role in some forms of human hypertension, adequate vitamin D levels may be important for decreasing the risk of high blood pressure.

Deficiency

In vitamin D deficiency, calcium absorption cannot be increased enough to satisfy the body's calcium needs (2). Consequently, PTH production by the parathyroid glands is increased and calcium is mobilized from the skeleton to maintain normal serum calcium levels—a condition known as secondary hyperparathyroidism. Although it has long been known that severe vitamin D deficiency has serious consequences for bone health, recent research suggests that less obvious states of vitamin D deficiency are common and increase the risk of osteoporosis and other health problems (16, 17).

Severe Vitamin D Deficiency

Rickets

In infants and children, severe vitamin D deficiency results in the failure of bone to mineralize. Rapidly growing bones are most severely affected by rickets. The growth plates of bones continue to enlarge, but in the absence of adequate mineralization, weight-bearing limbs (arms and legs) become bowed. In infants, rickets may result in delayed closure of the fontanelles (soft spots) in the skull, and the rib cage may become deformed due to the pulling action of the diaphragm. In severe cases, low serum calcium levels (hypocalcemia) may cause seizures. Although fortification of foods has led to complacency regarding vitamin D deficiency, nutritional rickets is still being reported in cities throughout the world (18, 19).

Osteomalacia

Although adult bones are no longer growing, they are in a constant state of turnover. In adults with severe vitamin D deficiency, the collagenous bone matrix is preserved but bone mineral is progressively lost, resulting in bone pain and osteomalacia (soft bones).

Muscle Weakness and Pain

Vitamin D deficiency causes muscle weakness and pain in children and adults. Muscle pain and weakness was a prominent symptom of vitamin D deficiency in a study of Arab and Danish Moslem women living in Denmark (20). In a cross-sectional study of 150 consecutive patients referred to a clinic in Minnesota for the evaluation of persistent, nonspecific musculoskeletal pain, 93% had serum 25(OH)D levels indicative of vitamin D deficiency (21). A randomized controlled trial found that supplementation of elderly women with 800 IU/day of vitamin D and 1,200 mg/day of calcium for three months increased muscle strength and decreased the risk of falling by almost 50% compared to supplementation with calcium alone (22).

Risk Factors for Vitamin D Deficiency

- **Excusively breast fed infants:** Infants who are exclusively breast fed and do not receive vitamin D supplementation are at high risk of vitamin D deficiency, particularly if they have dark skin and/or receive little sun exposure (19). Human milk generally provides 25 IU of vitamin D per liter, which is not enough for an infant if it is the sole source of vitamin D. Older infants and toddlers exclusively fed milk substitutes and weaning foods that are not vitamin D fortified are also at risk of vitamin D deficiency (18). The American Academy of Pediatrics recommends that all infants that are not consuming at least 500 ml (16 ounces) of vitamin D fortified formula or milk be given a vitamin D supplement of 200 IU/day

(19).

- **Dark skin:** People with dark skin synthesize less vitamin D on exposure to sunlight than those with light skin (1). The risk of vitamin D deficiency is particularly high in dark-skinned people who live far from the equator. In the U.S., 42% of African American women between 15 and 49 years of age were vitamin D deficient compared to 4% of White women (23).
- **Aging:** The elderly have reduced capacity to synthesize vitamin D in the skin when exposed to UVB radiation, and are more likely to stay indoors or use sunscreen. Institutionalized adults are at extremely high risk of vitamin D deficiency without supplementation (24, 25).
- **Covering all exposed skin or using sunscreen whenever outside:** Osteomalacia has been documented in women who cover all of their skin whenever they are outside for religious or cultural regions (26, 27). The application of sunscreen with an SPF factor of 8 reduces production of vitamin D by 95% (1).
- **Fat malabsorption syndromes:** Cystic fibrosis and cholestatic liver disease impair the absorption of dietary vitamin D (28).
- **Inflammatory bowel disease:** People with inflammatory bowel disease like Crohn's disease appear to be at increased risk of vitamin D deficiency, especially those who have had small bowel resections (29).
- **Obesity:** Obesity increases the risk of vitamin D deficiency (30). Once vitamin D is synthesized in the skin or ingested, it is deposited in body fat stores, making it less bioavailable to people with large stores of body fat.

Assessing Vitamin D Nutritional Status

Growing awareness that vitamin D insufficiency has serious health consequences beyond rickets and osteomalacia highlights the need for accurate assessment of vitamin D nutritional status. Although there is general agreement that the serum 25(OH)D level is the best indicator of vitamin D deficiency and sufficiency, the cutoff values have not been clearly defined (17). While laboratory reference ranges for serum 25(OH)D levels are often based on average values from populations of healthy individuals, recent research suggests that health-based cutoff values aimed at preventing secondary hyperparathyroidism and bone loss should be considerably higher. In general, serum 25 (OH)D values less than 20-25 nmol/L indicate severe deficiency associated with rickets and osteomalacia (16, 18). Although 50 nmol/L has been suggested as the low end of the normal range (31), more recent research suggests that PTH levels (32, 33) and calcium absorption (34) are not optimized until serum 25(OH)D levels reach approximately 80 nmol/L . Thus, at least one vitamin D expert has argued that serum 25(OH)D values less than 80 nmol/L should be considered deficient (16), while another suggests that a healthy serum 25(OH)D value is between 75 nmol/L and 125 nmol/L (35). Data from supplementation studies indicates that vitamin D intakes of at least 800-1,000 IU/day are required by adults living in temperate latitudes to achieve serum 25(OH)D levels of at least 80 nmol/L (36, 37).

The Adequate Intake (AI)

In 1997, the Food and Nutrition Board of the Institute of Medicine felt that the issue of sunlight exposure confounded the existing data on vitamin D requirements, making it impossible to calculate an RDA (28). Instead, the Food and Nutrition Board set adequate intake levels (AI) that assume no vitamin D is being synthesized in the skin through exposure to sunlight. The AI values established in 1997 (see table below) reflect vitamin D intakes likely to maintain serum 25(OH)D levels of at least 37.5 nmol/L, which many experts now feel is too low (2, 16, 17).

Adequate Intake (AI) for Vitamin D			
Life Stage	**Age**	**Males mcg/day (IU/day)**	**Females mcg/day (IU/day)**
Infants	0-6 months	5 mcg (200 IU)	5 mcg (200 IU)
Infants	7-12 months	5 mcg (200 IU)	5 mcg (200 IU)
Children	1-3 years	5 mcg (200 IU)	5 mcg (200 IU)
Children	4-8 years	5 mcg (200 IU)	5 mcg (200 IU)
Children	9-13 years	5 mcg (200 IU)	5 mcg (200 IU)
Adolescents	14-18 years	5 mcg (200 IU)	5 mcg (200 IU)
Adults	19-50 years	5 mcg (200 IU)	5 mcg (200 IU)
Adults	51-70 years	10 mcg (400 IU)	10 mcg (400 IU)
Adults	71 years and older	15 mcg (600 IU)	15 mcg (600 IU)
Pregnancy	all ages	-	5 mcg (200 IU)
Breastfeeding	all ages	-	5 mcg (200 IU)

Disease Prevention

Osteoporosis

Although osteoporosis is a multifactorial disease, vitamin D insufficiency can be an important contributing factor. Without sufficient vitamin D, calcium absorption cannot be maximized and the resulting elevation in PTH secretion by the parathyroid glands results in increased bone resorption, which may lead to osteoporotic fracture (38). A prospective cohort study that followed more than 72,000 postmenopausal women in the U.S. for 18 years found that those who consumed at least 600 IU/day of vitamin D from diet and supplements had a risk of osteoporotic hip fracture that was 37% lower than women who consumed less than 140 IU/day (39). The results of most clinical trials suggest that vitamin D supplementation can slow bone density losses or decrease the risk of osteoporotic fracture in men and women who are unlikely to be getting enough vitamin D.

Supplementation of postmenopausal women in the U.S. with 500 mg/day of calcium and either 100 IU/day or 700 IU/day of vitamin D for two years slowed bone density losses at the hip only in the group taking 700 IU/day (40). Daily supplementation of elderly men and women with 500 mg/day of calcium and 700 IU/day of vitamin D for three years reduced bone density losses at the hip and spine and reduced the frequency of nonvertebral fractures (41). When the calcium and vitamin D supplements were discontinued, the bone density benefits were lost within 2 years (42). In Denmark, supplementation of elderly women with 400 IU/day of vitamin D for two years increased bone density at the hip (43).

An annual injection of 150,000-300,000 IU of vitamin D2 (ergocalciferol) for four years decreased the incidence of fracture in elderly Finnish women (44), and oral supplementation with 800 IU/day of vitamin D and 1,200 mg/day of calcium for three years decreased the incidence of hip fracture in elderly French women (45). Oral

supplementation of elderly adults in the U.K. with 100,000 IU of vitamin D once every four months (equivalent to about 800 IU/day) for five years reduced the risk of osteoporotic fracture by 33% compared to placebo (46). However, oral supplementation with 400 IU/day of vitamin D for more than 3 years did not affect the incidence of fracture in a study of elderly Dutch men and women (47). Overall, the evidence to date suggests that vitamin D supplements of about 800 IU/day may be helpful in reducing bone loss and fracture rates in the elderly. In order for vitamin D supplementation to be effective in preserving bone health, adequate calcium (1,000 to 1,200 mg/day) should also be consumed.

Cancer

Two characteristics of cancer cells are their lack of differentiation (specialization) and their rapid growth or proliferation. Many malignant tumors have been found to contain vitamin D receptors (VDR), including breast, lung, skin (melanoma), colon, and bone. Biologically active forms of vitamin D, such as 1,25(OH)2D and its analogs, have been found to induce cell differentiation and/or inhibit proliferation of a number of cancerous and noncancerous cell types maintained in cell culture (48).

Colorectal Cancer

The geographic distribution of colon cancer is similar to the historic geographic distribution of rickets, providing circumstantial evidence that decreased sunlight exposure and diminished vitamin D nutritional status may be related to an increased risk of colon cancer. However, prospective cohort studies have not generally found total vitamin D intake to be associated with significant reductions in colorectal cancer when other risk factors are taken into account (49-52). One five-year study of more than 120,000 people found that men with the highest vitamin D intakes had a risk of colorectal cancer that was 29% lower than men with the lowest vitamin D intakes (53). Vitamin D intake was not significantly associated with colorectal cancer risk in women. Serum 25(OH)D level, which reflects vitamin D intake and vitamin D synthesis, was inversely associated with the risk of potentially precancerous colorectal polyps (54) and indices of colonic epithelial cell proliferation, (55) which are considered biomarkers for colon cancer risk.

Breast Cancer

Although breast cancer mortality follows a similar geographic distribution to that of colon cancer (56, 57), direct evidence of an association between vitamin D nutritional status and breast cancer risk is limited.. A prospective study of women who participated in the first National Health and Nutrition Examination Survey (NHANES I) found that several measures of sunlight exposure and dietary vitamin D intake were associated with a reduced risk of breast cancer 20 years later (58). More recently, a 16-year study of more than 88,000 women found that higher intakes of vitamin D were associated with significantly lower breast cancer risk in premenopausal women but not postmenopausal women (59).

Prostate Cancer

Epidemiological studies show correlations between risk factors for prostate cancer and conditions that can result in decreased vitamin D levels (48). Increased age is associated with an increased risk of prostate cancer, as well as with decreased sun exposure and decreased capacity to synthesize vitamin D. The incidence of prostate cancer is higher in African American men than in White American men, and the high melanin content of

dark skin is known to reduce the efficiency of vitamin D synthesis. Geographically, mortality from prostate cancer is inversely associated with the availability of sunlight. Recent findings that prostate cells in culture can synthesize the 25(OH)D3-1-hydroxylase enzyme and that, unlike the renal enzyme, its synthesis is not influenced by PTH or calcium levels also provide support for the idea that increasing 25(OH)D levels may be useful in preventing prostate cancer (60). In contrast, prospective studies have not generally found significant relationships between serum 25(OH)D levels and subsequent risk of developing prostate cancer (61-64). Although a prospective study of Finnish men found that low serum 25(OH)D levels were associated with earlier and more aggressive prostate cancer development (65), another prospective study of men from Finland, Norway and Sweden found a U-shaped relationship between serum 25(OH)D levels and prostate cancer risk. In that study serum 25(OH)D concentrations of 19 nmol/L or lower and 80 nmol/L or higher were associated with higher prostate cancer risk (66). Further research is needed to determine the nature of the relationship between vitamin D nutritional status and prostate cancer risk.

Autoimmune Diseases

Insulin-dependent diabetes mellitus (IDDM), multiple sclerosis (MS), and rheumatoid arthritis (RA) are each examples of autoimmune disease. Autoimmune diseases occur when the body mounts an immune response to its own tissue, rather than a foreign pathogen. In IDDM, insulin producing beta-cells of the pancreas are the target of the inappropriate immune response. In MS, the targets are the myelin producing cells of the central nervous system, and in RA, the targets are the collagen producing cells of the joints (67). Autoimmune responses are mediated by immune cells called T cells. The biologically active form of vitamin D, 1,25(OH)2D, has been found to modulate T cell responses, such that the autoimmune responses are diminished. Treatment with 1,25(OH) 2D has beneficial effects in animal models of IDDM , MS, and RA. epidemiological studies have found that the prevalence of IDDM, MS, and RA increases as latitude increases, suggesting that lower exposure to UVB radiation and associated decreases in vitamin D synthesis may play a role in the pathology of these diseases. The results of several prospective cohort studies also suggest that adequate vitamin D intake may decrease the risk of autoimmune diseases. A prospective cohort study of children born in Finland during the year 1966 and followed for thirty years found that those who received vitamin D supplementation in the first year of life had a significantly lower risk of developing IDDM, while children suspected of developing rickets (severe vitamin D deficiency) during the first year of life had a significantly higher risk of developing IDDM (68). Vitamin D supplement use was associated with a significant reduction in the risk of developing MS in two large cohorts of women followed for at least ten years (69). Similarly, postmenopausal women with the highest total vitamin D intakes were at significantly lower risk of developing RA after eleven years of follow up than those with the lowest intakes (70). Evidence from animal models and epidemiological studies suggests that maintaining sufficient vitamin D levels may help decrease the risk of several autoimmune diseases.

Hypertension (High Blood Pressure)

The results of epidemiological and clinical studies suggest an inverse relationship between serum 1,25(OH)2D levels and blood pressure, which may be explained by recent findings that 1,25(OH)2D decreases the expression of the gene encoding renin (see Function). Data from epidemiological studies suggest that conditions that decrease vitamin D synthesis in the skin, such as having dark skin and living in temperate latitudes, are associated with increased prevalence of hypertension (71). A controlled clinical trial

in 18 hypertensive men and women living in the Netherlands found that exposure to UVB radiation three times weekly for six weeks during the winter increased serum 25(OH)D levels and significantly decreased 24-hour ambulatory systolic and diastolic blood pressure measurements by an average of 6 mm Hg (72). In randomized controlled trials of vitamin D supplementation, a combination of 1,600 IU/day of vitamin D and 800 mg/day of calcium for eight weeks significantly decreased systolic blood pressure in elderly women by 9% compared to calcium alone (73), but supplementation with 400 IU/day or a single dose of 100,000 IU of vitamin D did not significantly lower blood pressure in elderly men and women over the next two months (74, 75). At present, data from controlled clinical trials are too limited to determine whether vitamin D supplementation will be effective in lowering blood pressure or preventing hypertension.

Sources

Sunlight

Sunlight exposure provides most people with their entire vitamin D requirement. Children and young adults who spend a short time outside two or three times a week will generally synthesize all the vitamin D they need. The elderly have diminished capacity to synthesize vitamin D from sunlight exposure and frequently use sunscreen or protective clothing in order to prevent skin cancer and sun damage. The application of sunscreen with an SPF factor of 8 reduces production of vitamin D by 95%. In latitudes around 40 degrees north or 40 degrees south (Boston is 42 degrees north), there is insufficient UVB radiation available for vitamin D synthesis from November to early March. Ten degrees farther north or south (Edmonton, Canada) this "vitamin D winter" extends from mid October to mid March. According to Dr. Michael Holick, as little as 5-10 minutes of sun exposure on arms and legs or face and arms three times weekly between 11:00 am and 2:00 pm during the spring, summer, and fall at 42 degrees latitude should provide a light-skinned individual with adequate vitamin D and allow for storage of any excess for use during the winter with minimal risk of skin damage (35).

Food sources

Vitamin D is found naturally in very few foods. Foods containing vitamin D include some fatty fish (mackerel, salmon, sardines), fish liver oils, and eggs from hens that have been fed vitamin D. In the U.S., milk and infant formula are fortified with vitamin D so that they contain 400 IU (10 mcg) per quart. However, other dairy products such as cheese and yogurt are not always fortified with vitamin D. Some cereals and breads are also fortified with vitamin D. Recently, orange juice fortified with vitamin D has been made available in the U.S. Accurate estimates of average dietary intakes of vitamin D are difficult because of the high variability of the vitamin D content of fortified foods (28). Vitamin D contents of some vitamin D-rich foods are listed in the table below in both international units (IU) and micrograms (mcg). For more information on the nutrient content of foods you eat frequently, search the USDA food composition database.

Food	Serving	Vitamin D (IU)	Vitamin D (mcg)
Pink salmon, canned	3 ounces	530	13.3
Sardines, canned	3 ounces	231	5.8
Mackerel, canned	3 ounces	214	5.4
Quaker Nutrition for Women			

Instant Oatmeal	1 packet	140	3.5
Cow's milk, fortified with vitamin D	8 ounces	100	2.5
Orange juice, fortified with vitamin D	8 ounces	100	2.5
Cereal, fortified	1 serving (usually 1 cup)	40-50	1.0-1.3
Egg yolk	1 medium	25	0.63

Supplements

Most vitamin D supplements available without a prescription contain cholecalciferol (vitamin D3). Multivitamin supplements for children generally provide 200 IU (5 mcg) and multivitamin supplements for adults generally provide 400 IU (10 mcg) of vitamin D. Single ingredient vitamin D supplements may provide 400-1,000 IU of vitamin D, but 400 IU is the most commonly available dose. A number of calcium supplements may also provide vitamin D.

Safety

Toxicity

Vitamin D toxicity (hypervitaminosis D) induces abnormally high serum calcium levels (hypercalcemia), which could result in bone loss, kidney stones, and calcification of organs like the heart and kidneys if untreated over a long period of time. When the Food and Nutrition Board of the Institute of Medicine established the tolerable upper intake level (UL) for vitamin D, published studies that adequately documented the lowest intake levels of vitamin D that induced hypercalcemia were very limited. Because the consequences of hypercalcemia are severe, the Food and Nutrition Board established a very conservative UL of 2,000 IU/day (50 mcg/day) for children and adults (see table below) (28). Research published since 1997 suggests that the UL for adults is likely overly conservative and that vitamin D toxicity is very unlikely in healthy people at intake levels lower than 10,000 IU/day (36, 76, 77). Vitamin D toxicity has not been observed to result from sun exposure. Certain medical conditions can increase the risk of hypercalcemia in response to vitamin D, including primary hyperparathyroidism, sarcoidosis, tuberculosis, and lymphoma (36). People with these conditions may develop hypercalcemia in response to any increase in vitamin D nutrition and should consult a qualified health care provider regarding any increase in vitamin D intake.

Tolerable Upper Intake Level (UL) for Vitamin D	
Age Group	**mcg/day (IU/day)**
Infants 0-12 months	25 mcg (1000 IU)
Children 1-18 years	50 mcg (2000 IU)
Adults 19 years and older	50 mcg (2000 IU)

Drug interactions

The following medications increase the metabolism of vitamin D and may decrease serum 25(OH)D levels: phenytoin (Dilantin), fosphenytoin (Cerebyx), phenobarbitol (Luminal), carbamazepine (Tegretol), and rifampin (Rimactane). The following medications should not be taken at the same time as vitamin D because they can decrease the intestinal absorption of vitamin D: cholestyramine (Questran), colestipol (Colestid), orlistat (Xenical), mineral oil, and the fat substitute Olestra. The oral anti-fungal medication, ketoconazole, inhibits the 25(OH)D3-1-hydroxylase enzyme and has been found to reduce serum levels of 1,25(OH)D in healthy men . The induction of hypercalcemia by toxic levels of vitamin D may precipitate cardiac arrhythmia in patients on digitalis (Digoxin) (78, 79).

The Linus Pauling Institute Recommendation

The Linus Pauling Institute recommends that generally healthy adults take a multivitamin supplement that supplies 400 IU (10 mcg) of vitamin D daily. Additionally, at least 10-15 minutes of sun exposure on the arms and legs or face and arms at least three times weekly between 11:00 am and 2:00 pm during the spring, summer, and fall may help residents of temperate latitudes (much of the U.S.) avoid vitamin D deficiency at the end of winter.

Older adults (65 years and older) and people with minimal sun exposure

In addition to the 400 IU (10 mcg) of vitamin D provided by a multivitamin supplement, people over the age of 65 and people who get minimal sun exposure throughout the year should take an additional vitamin D supplement of 400 day (10 mcg/day) to provide a total of 800 IU/day (20 mcg/day).

References

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CHAPTER 89

Vitamin D: Cancer and Differentiation

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I. INTRODUCTION

The seco-steroid hormone 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] is the most potent natural metabolite of vitamin D_3 and is an important regulator of calcium homeostasis and bone metabolism via actions in intestine, bone, kidney, and parathyroid glands. 1,25-$(OH)_2D_3$ exerts its effects via an intracellular receptor that is a member of the steroid hormone receptor family (see Chapters 11–20 and 22 in this book). Throughout the last decades, it has become evident that the vitamin D receptor (VDR) is not limited to cells and tissues involved in regulation of calcium and bone metabolism but is also present in a wide variety of other cells and tissues including cancer cells of various origins. This led to a vast series of studies on the role of vitamin D in tumor cell growth regulation, treatment of cancer, and development of potent synthetic vitamin D analogs. Various specialized chapters will discuss in detail the effect of vitamin D on specific cancers (Chapters 89–97) and development and actions of vitamin D analogs (Chapters 80–88). In this chapter we aim to give an overview of the history and current stage and developments on vitamin D and cancer, regulation of tumor cells, possible mechanisms, and clinical applications.

II. VITAMIN D AND CANCER

A. Vitamin D Receptor

As exemplified in Table I, the VDR has also been demonstrated in a broad range of tumors and malignant cell types. For colon and breast cancer cells, an inverse relationship between VDR level and degree of differentiation has been described by some investigators [1,2]. VDR level is increased in ovarian carcinoma compared to normal ovarian tissue [3]. For colorectal cancer it was shown that VDR expression is associated with a more favorable prognosis in colorectal cancer [4]. A VDR immunoreactivity score showed an increase in breast carcinoma specimens compared to normal breast tissue but no clear relation with proliferative status could be assessed [5]. A later study by the same group showed that VDR expression is not a prognostic factor for breast cancer, but the strong VDR immunoreactivity in the breast cancer specimens supports the evidence for it to be a target for intervention [6]. Also in other studies no associations between VDR and clinical and biochemical parameters of breast cancer were found [7–12].

Albeit that the association studies on VDR expression and predictive and/or prognostic characteristics for cancer are so far not conclusive, the widespread distribution of the VDR in malignant cells indicates that regulation of cancer cell function might be a new target in the action of 1,25-$(OH)_2D_3$ and provides a biological basis for the epidemiological observations discussed in the next paragraph.

A recent observation put the VDR in relation to cancer in a whole new perspective. It was shown that VDR can function as a receptor for the secondary bile acid lithocholic acid. This compound is hepatotoxic and a potential enteric carcinogenic. Interestingly, both binding of lithocholic acid and vitamin D to the VDR results in induction of CYP3A, the enzyme that detoxifies lithocholic acid in the liver and intestine [13,14]; (see also Chapter 53). It is postulated that vitamin D and lithocholic acid, by binding to the VDR, activate a feed-forward catabolic pathway that increases CYP3A expression leading to detoxification of carcinogenic bile acids. A relation between the presence of VDR and carcinogenesis was recently also shown for the skin. Absence of VDR increased the sensitivity for chemically induced tumorigenesis [15].

B. Epidemiology

In 1980 an epidemiological study based on indirect evidence suggested a relationship between vitamin D and cancer. This was derived from analyses of death

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TABLE I VDR in Tumors and Malignant Cell Types

Basal cell carcinoma	Myeloid leukemia
Breast carcinoma	Myeloma
Bladder cancer	Osteogenic sarcoma
Cervical carcinoma	Ovarian carcinoma
Colonic adenocarcinoma	Neuroblastoma
Colorectal carcinoma	Non-Hodgkin's lymphoma
Gall bladder carcinoma	Pancreatic carcinoma
Glioma cells	Parathyroid adenoma
Kaposi sarcoma	Pituitary adenoma
Lung carcinoma	Prostate carcinoma
Lymphocytic leukemia	Renal cell carcinoma
Malignant B-cell progenitors	Squamous cell carcinoma
Malignant melanoma	Transitional cell bladder carcinoma
Medullary thyroid carcinoma	Uterine carcinosarcoma

rates from colon cancer, which tended to increase with increasing latitude and decreasing sunlight [16]. Later more direct evidence about a relation between vitamin D and colon cancer came from the inverse relationship between levels of serum 25-hydroxyvitamin D_3 [a 1,25-$(OH)_2D_3$ precursor] and incidence of colonic cancer [17,18]. In addition, a similar relationship between sunlight exposure, vitamin D, and the risk for fatal breast and prostate cancer has been suggested [19–23] (see Chapter 90). The relationship between sunlight exposure and cancer, especially with respect to vitamin D, has been carefully reviewed by Studzinski and Moore [24]. The dual relationship between sunlight and cancer is of interest and remains the subject of continuing studies [25–27]. A relationship between skin type and prostate cancer has been described [28–30] and recently an article on the skin, sunlight, vitamin D, and cancer has been presented from an evolutionary perspective [31].

The relationship between cancer, diet, and calcium intake and vitamin D has been addressed in several studies [32–37] (see Chapter 91). A Canadian study noted similar vitamin D intakes in breast cancer patients and control subjects [38]. Moreover, in a mouse model, no relationship was found between dietary intake of a wide range of doses of calcium or vitamin D and carcinogen-induced skin tumors [39]. A large Finish epidemiological study showed an association of low serum 25-hydroxy-vitamin D_3 with prostate cancer [40,41]. A study on intake of micronutrients suggested that vitamin D and calcium might interact with antioxidants like vitamin C and E in reducing colorectal cancer risk [42]. It is clear that sunlight exposure, vitamin D intake, and other dietary components such as calcium and fat should be considered as possibly interacting with one another when the relationship between vitamin D and cancer risk is assessed. The data on VDR as bile acid sensor and its postulated role in detoxification provide a direct biological basis for the relation between increased colon cancer and high-fat diets [43] and that colon cancer occurs in areas with higher prevalence of rickets [36]. In addition, mice lacking VDR have been reported to have a higher proliferation rate in the colon [44,45]. A survey of mutations in the VDR in osteosarcomas, several other sarcomas, nonsmall cell lung cancers, and a large number of cell lines representing many tumor types did not show that mutations or rearrangements in the VDR gene play a role in these cancers [46]. Aspects on sunlight and the epidemiology of vitamin D and calcium will be further discussed in greater detail in Chapters 90 and 91, respectively.

In the VDR gene several polymorphisms have been identified and studied in relation to various endpoints (discussed in Chapter 68). Throughout the last years, an increasing number of studies have studied the association of polymorphisms in the VDR and cancer. The first study showed an association between polymorphisms at the 3′ end of the VDR gene and prostate cancer [47]. This was shortly followed by a study showing an association of prostate cancer with variations in the 3′ poly-A stretch in the VDR gene [48]. Interestingly, the Odds Ratio for the VDR polymorphism was about twofold that of the one for the CAG repeat in the androgen receptor. This was followed by several others studies also showing associations of polymorphisms in the 3′ region of the VDR gene and prostate cancer, [49–55] albeit other studies couldn't confirm this [56–60]. For breast cancer both presence [61–66] and absence of association [67] with polymorphisms in the VDR gene have been reported. Also for colon cancer both presence [68,69] and absence [70] of an association with VDR polymorphisms have been reported. No association was reported with basal cell carcinoma [71]. A single study reported an association with the aggressive renal cell carcinoma [72], malignant melanoma [73], and another study on rectal cancer reported a correlation between VDR gene polymorphisms and erbB-2/HER-2 expression [74]. It should be concluded that so far the studies on VDR gene polymorphisms and cancer are far from conclusive. A major reason might be the limited size of most of the studies. More association studies on VDR gene polymorphisms and specific cancers are needed, which should be followed by a meta-analysis to definitively assess whether there is an association and if so, what is the size of the effect. Also, for studies on VDR gene polymorphisms, it is important to take into account the

impact of environmental factors. Diet, vitamin D intake, and sun exposure may modify the association of polymorphism and cancer risk. Interaction between vitamin D and calcium intake and cancer was also found in some of the VDR gene polymorphism studies [68,75–77]. Some studies reported decreased risk of prostate cancer [75] and colorectal adenomas [76] in those subjects with lower vitamin D levels and a particular VDR gene polymorphism. However, results of these studies are unusual in light of the fact that higher calcium and vitamin D intake are generally associated with a modestly reduced risk of colorectal neoplasia. Finally, most importantly it should be realized that except for the FokI translational start site polymorphism, all polymorphisms analyzed so far are anonymous, and functionality or linkage with functional polymorphisms should be proven. The 3′ polymorphisms have been shown to be in linkage with 3′-UTR polymorphisms, but no relation with VDR mRNA stability could be proven [78]. Detailed discussion of possible functional consequences of VDR gene polymorphisms and impact of vitamin D levels is beyond the scope of this chapter but will be addressed in Chapter 67.

C. Growth and Development

In addition to the epidemiological studies and demonstration of vitamin D receptor in tumor cells, since the early 1980s there has also been an increasing amount of cell biological data supporting a role for vitamin D in cancer. Multiple studies have shown that at high concentrations (10^{-9}–10^{-7} M) 1,25-$(OH)_2D_3$ inhibits the growth of tumor cells *in vitro*. It was demonstrated as early as 1981 that 1,25-$(OH)_2D_3$ inhibits the growth of malignant melanoma cells and stimulates the differentiation of immature mouse myeloid leukemia cells in culture [79–81]. 1,25-$(OH)_2D_3$ also induces differentiation of normal bone marrow cells (see Chapter 96). Immature bone marrow cells of the monocyte-macrophage lineage are believed to be the precursors of osteoclasts, and 1,25-$(OH)_2D_3$ induces differentiation of immature myeloid cells toward monocytes-macrophages and also stimulates the activation and fusion of some macrophages (discussed in Chapter 38). From these results, it has been postulated that 1,25-$(OH)_2D_3$ stimulates differentiation and fusion of osteoclast progenitors into osteoclasts [82–84]. Also, in the intestine, 1,25-$(OH)_2D_3$ has important effects on cellular proliferation and differentiation [85]. Thus, via stimulation of the differentiation inducing capacity of bone and interstitial cells, 1,25-$(OH)_2D_3$ may play an important role in the regulation of calcium and bone metabolism. These *in vitro* findings were followed by the *in vivo* observation that 1,25-$(OH)_2D_3$ prolongs the survival time of mice inoculated with myeloid leukemia cells [86]. As shown in Table II, over the years 1,25-$(OH)_2D_3$ has been shown to have beneficial effects in several other *in vivo* animal models of various types of cancers [87–109].

An important aspect and limitation of the treatment of cancer with 1,25-$(OH)_2D_3$ was revealed by this limited set of clinical trials (see section II.D); to achieve growth inhibition, high doses are needed (confirming the *in vitro* data), which can cause the side effect of hypercalcemia. This has prompted the development of analogs of 1,25-$(OH)_2D_3$ in order to dissociate the antiproliferative effect from the calcemic and bone metabolism effects (see Chapters 80–88) [110,111]. Although the precise mechanism is not completely understood, at the moment several 1,25-$(OH)_2D_3$ analogs are available that seem to fulfill these criteria. In Table III the *in vivo* animal studies using 1,25-$(OH)_2D_3$ analogs on various cancer types are summarized [97,103,104,106–109,112–129].

D. Clinical Studies

Considering the calcemic actions of 1,25-$(OH)_2D_3$ up to this point in time only a few clinical trials of vitamin D compounds in cancer have been performed. Alfacalcidol (1α-hydroxyvitamin D_3; 1α-$(OH)D_3$), which is converted to 1,25-$(OH)_2D_3$ *in vivo*, caused a beneficial response in low-grade non-Hodgkin's lymphoma patients [130,131]. Also, with alfacalcidol, transient improvement in peripheral blood counts was seen in patients with myelodysplasia; however, half of the patients developed hypercalcemia [132]. Another study reported a sustained hematological response in six myelodysplasia patients treated with high doses of alfacalcidol [133]. These patients were restricted in their dietary calcium intake; nevertheless, four patients developed hypercalcemia due to increased bone resorption. With respect to treatment of cutaneous T-cell lymphoma with a combination of 1,25-$(OH)_2D_3$ and retinoids, contrasting results have been obtained. It has been suggested that the variability was due to differences in phenotype of the various lymphomas [134–138]. A study on early recurrent prostate cancer showed that daily treatment with 1,25-$(OH)_2D_3$ slowed the rise in prostate-specific antigen, but treatment coincided with hypercalcemic affects [139]. Using a regime of weekly treatment with high-dose calcitriol was found to be safe, but didn't result in a significant reduction in prostate-specific antigen (PSA) in prostate cancer cells [140]. Two studies were specifically designed to examine the route of application and calcemic response in patients with advanced malignancies [141,142].

TABLE II *In Vivo* Effects of 1,25-$(OH)_2D_3$ and 1α-$(OH)D_3$ in Animal Models of Cancer[a]

Tumor	Model	Effect	Refs.
Adenocarcinoma	CAC-8 cells injected in nude mice	Reduction in tumor volume	[107]
Breast	NMU- and DMBA-induced breast cancer in rats	Tumor suppression	[93,96]
Colon	Human colon cell line implanted into nude mice; DMH-induced colon cancer in rats; APCmin mice	Tumor suppression; reduction of the incidence of colon adenocarcinomas; decrease in polyp number and tumor load	[90,92,95,371]
Kaposi sarcoma	KS Y-1 cells implanted in nude mice	Tumor growth retardation	[105]
Leydig tumor	Leydig cell tumor implanted into rats	Tumor suppression	[97]
Lung	Implantation of lewis lung carcinoma into mice	Reduction of the number of metastases (without suppression of primary tumor); tumor suppression; increased antitumor immunity	[87,99,101,102]
Melanoma	Human melanoma cells implanted into nude mice	Tumor suppression	[90]
Osteosarcoma	Human osteosarcoma cells implanted into nude mice	Tumor suppression	[98]
Prostate	Dunning MAT LyLu rat prostate model; LNCaP xenografts in nude mice; PAIII tumors in Lobund-Wistar rats	Reduction in lung metastasis; tumor suppression	[103,104,106,108,109]
Retinoblastoma	Retinoblastoma cell line implanted into nude mice; transgenic mice with retinoblastoma	Tumor suppression	[91,94]
Walker carcinoma	Walker carcinoma cells injected in rats	Tumor suppression	[100]
Skin	DMBA/TPA-induced skin tumors in mice	Inhibition of tumor formation	[88,89]

[a]The dosage, duration of treatment, diet, and effects on serum/urinary calcium vary among the studies. NMU, Nitrosomethylurea; DMBA, 7,12-dimethylbenz[a]anthracene; DMH, 1,2-dimethylhydrazine dihydrochloride; TPA, 12-O-tetradecanoylphorbol-13-acetate.

Clinical trials using vitamin D analogs have been initiated over the last years. However, these were mostly limited clinical trials focusing on small groups of patients for whom regular treatment has failed. Only data from a few studies has been published. The analog calcipotriol (MC903) has been used for topical treatment of advanced breast cancer; however, several of the patients still developed hypercalcemia [143]. More recent studies have been published on advanced breast cancer [144] and pancreatic cancer [145] but the clinical results were limited. In a single case of Kaposi sarcoma and topical application of calcipotriol (Daivonex/Dovonex/MC903), good success in tumor regression was reported [105]. In Chapter 97 the current clinical status of 1,25-$(OH)_2D_3$ and its analogs as therapeutic agents for cancer will be discussed in greater detail.

E. Angiogenesis and Metastasis

For the tumor suppressive activity of vitamin D_3 compounds *in vivo*, besides growth inhibition, two additional actions may be involved. First, angiogenesis is an essential requirement for the growth of solid tumors. Compounds that inhibit angiogenesis might therefore contribute to antitumor therapy. Antiangiogenic drugs may cause inhibition of tumor progression, stabilization of tumor growth, tumor regression, and prevention of metastasis. Antiangiogenic effects may play a role in the tumor suppressive activity of vitamin D_3 compounds. Two studies reported an antiangiogenic effect of 1,25-$(OH)_2D_3$ and the analog 22-oxacalcitriol using different experimental model systems [115,146]. In addition, it was shown that 1,25-$(OH)_2D_3$ inhibits angiogenesis induced by the human papilloma virus type 16 (HPV16)- or HPV18-containing cell lines HeLa, Skv-e2, and Skv-el2 when intradermally injected into immunosuppressed mice [147]. Also, with the non-virus-transformed human cell lines T47-D (breast carcinoma) and A431 (vulva carcinoma), similar results were obtained [148]. In these studies the mice were treated for 5 days with 1,25-$(OH)_2D_3$ prior to the injection of tumor cells. The effect of 1,25-$(OH)_2D_3$ on angiogenesis may be due to inhibition of tumor cell proliferation, resulting in fewer angiogenic cells.

TABLE III *In Vivo* Effects 1,25-$(OH)_2D_3$ Analogs in Animal Models for Cancer[a]

Analog	Model	Antitumor effect	Refs.
1,25-$(OH)D_2$	Retinoblastoma	Tumor suppression	[128]
1,25-$(OH)D_5$	Breast	Tumor suppression	[129]
CB966	Breast	Tumor suppression	[114]
CB1093	Prostate	Tumor suppression	[108]
		No effect on angiogenesis	
DD-003	Colon	Tumor suppression	[120]
EB1089	Adenocarcinoma	Tumor suppression	[107]
EB1089	Breast	Tumor suppression	[114,116,125,316]
EB1089	Colon	Tumor suppression	[124]
EB1089	Hepatocellular carcinoma	Inhibition of tumor incidence	[372]
EB1089	Leydig cell tumor	Tumor suppression	[97]
EB1089	Prostate	Tumor suppression	
		Reduction lung metastases	[104,106,108,109,126,127]
		No effect on angiogenesis	
KH1060	Prostate	Tumor suppression	[109]
LG190119	Prostate	Tumor suppression	[106]
OCT	Breast	Tumor suppression	[113,118]
OCT	Breast	Tumor suppression	[115]
OCT	Breast	Tumor suppression	[118]
OCT	Colon	Decreased tumor incidence	[121]
MC903	Breast	Tumor suppression	[117]
Ro 23-7553	Prostate	Tumor suppression	[122]
Ro 23-7553	Leukemia	Increased survival	[112]
Ro 24-5531	Breast	Decreased tumor incidence	[119]
Ro 24-5531	Colon	Decreased tumor incidence	[123]
Ro-25-6760	Prostate	Tumor suppression	[103]
Ro-26-9114	Colon	Decrease in polyp number and tumor load	[371]
Ro-26-9114	Prostate	Tumor suppression	[109]

[a]MC903, 1,24-dihydroxy-22-ene-24-cyclopropyl-vitamin D_3; CB966, 24a,26a,27a-tri-homo-1α,25-dihydroxyvitamin D_3; CB1093, 20-epi-22(S)-ethoxy-23yne-24a, 26a,27a-trihomo-1α,25-dihydroxyvitamin D_3; DD-003,22(S)-24-homo-26,26,26,27,27,27-hexafluoro-1α,22,25-trihydroxyvitamin D_3; EB1089, 22,24-diene-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3; OCT, 22-Oxacalcitriol; Ro 23-7553, 1,25-dihydroxy-16-ene-23-yne-vitamin D_3; Ro 24-5531, 1,25-dihydroxy-16-ene-23-yne-26,27-hexafluorovitamin D_3. Ro 26-9114, 1α,25-$(OH)_2$-16-ene-19-nor-24-oxo-D_3.

However, inhibition of angiogenesis could also be observed when the tumor cells were treated *in vitro* with 1,25-$(OH)_2D_3$ and, after cell washing, were injected into mice [148]. Under these conditions both control and 1,25-$(OH)_2D_3$-treated mice were injected with similar numbers of cells. Therefore, these data indicate that 1,25-$(OH)_2D_3$ inhibits the release of angiogenic factors (vascular endothelium growth factor, transforming growth factor-α, basic fibroblast growth factor, epidermal growth factor, etc.) or stimulates antiangiogenic factors. 1,25-$(OH)_2D_3$ treatment caused a reduction in the angiogenic signaling molecule, angiopoietin-2 in squamous cell carcinoma and radiation-induced fibrosarcoma-1 cells [149]. In retinoblastomas in mice, 1,25-$(OH)_2D_3$ has also been shown to reduce angiogenesis [150]. A recent study by Oades *et al.*, however, showed that the 1,25-$(OH)_2D_3$ analogs EB1089 and CB1093 inhibited tumor growth in two prostate animal models but did not inhibit angiogenesis in a rat aorta assay [108]. Whether this indicates that vitamin D affects angiogenesis in a tumor situation and not in a nonmalignant condition is not clear. This may resemble the effects of endostatin, which inhibits pathological but not normal

vascularization [151,152]. In support of this is the finding that 1,25-$(OH)_2D_3$ and its analogs EB1089, Ro-25-6760, and ILX23-7553 potently inhibit growth of endothelial cells derived from tumors, but are less potent against normal aortic or yolk sac endothelial cells [149]. Finally, an interesting observation is deglycosylated vitamin D–binding protein (DBP-maf) has also been reported to inhibit angiogenesis [153,154] and to inhibit growth of pancreatic tumor in nude mice [154]. Whether 1,25-$(OH)_2D_3$ may interfere with DBP-maf in tumor growth inhibition and antiangiogenesis remains to be established. Interaction with another factor, interleukin-12, in the inhibition of angiogenesis has been reported [155].

The second mechanism of antitumor activity, which may be related to angiogenesis, is metastasis. Metastasis is the primary cause of the fatal outcome of cancer diseases. A study by Mork Hansen *et al.* indicated that 1,25-$(OH)_2D_3$ may be effective in reducing the invasiveness of breast cancer cells [156]. They showed that 1,25-$(OH)_2D_3$ inhibited the invasion and migration of a metastatic human breast cancer cell line (MDA-MB-231) using the Boyden chamber invasion assay. In support of this, it was shown that 1,25-$(OH)_2D_3$, KH1060, EB1089, and CB1093 inhibited secretion of tissue-type and urokinase plasminogen activator and increased plasminogen activator inhibitor 1 in the MDA-MB-231 metastatic breast cancer cells [157]. In an *in vivo* study, it was shown that 1,25-$(OH)_2D_3$ reduces metastasis to the lung of subcutaneously implanted Lewis lung carcinoma cells [101]. In two animal models of prostate cancer, 1,25-$(OH)_2D_3$ and the analogs EB1089 and RO25-6760 inhibited lung metastases [103,104]. In these models, the tumors were implanted subcutaneously and therefore, in contrast to the model of direct tumor cell injection in the left ventricle [158], no bone metastases occurred. However, a fact to be considered in relation to metastasis is that bone is the most frequent site of metastasis of advanced breast and prostate cancer. There are some indications from clinical studies that bone metastases develop preferentially in areas with high bone turnover [159,160]. In contrast, agents that inhibit bone resorption have been reported to reduce the incidence of skeletal metastasis [161]. As 1,25-$(OH)_2D_3$ may stimulate bone turnover, treatment of cancer with 1,25-$(OH)_2D_3$ might theoretically increase the risk of skeletal metastases. This aspect of 1,25-$(OH)_2D_3$ therapy certainly needs further study. In this aspect, the use of vitamin D_3 analogs with reduced calcemic activity or treatment with vitamin D_3 in combination with other compounds to reduce bone turnover (see Section IV) may be helpful. The data obtained so far on angiogenesis and metastasis indicate that these two processes are part of the spectrum of mechanisms by which vitamin D_3 exerts its anticancer activity.

F. Parathyroid Hormone-Related Peptide

1,25-$(OH)_2D_3$ and parathyroid hormone (PTH) mutually regulate synthesis and secretion of one another. Production and secretion of PTH are inhibited by 1,25-$(OH)_2D_3$ via a transcriptional effect, and a vitamin D responsive element (VDRE) in the promoter of the PTH gene has been identified [162,163] (see Chapter 30). Parathyroid hormone-related peptide (PTHrP) was initially isolated from several carcinomas and is responsible for the humoral hypercalcemia of malignancy syndrome [164]. Although originally identified in carcinomas, PTHrP has also been identified in normal cells (see Chapter 43).

In normal human mammary epithelial cells, 1,25-$(OH)_2D_3$ did not affect basal but inhibited growth factor-stimulated PTHrP expression via an effect on transcription [165]. In normal keratinocytes 1,25-$(OH)_2D_3$ had no effect on PTHrP secretion in basal culture conditions [166], but did inhibit growth factor-stimulated PTHrP production as well [167]. Likewise, 1,25-$(OH)_2D_3$, as well as the analogs 22-oxacalcitriol and MC903, inhibited PTHrP secretion in immortalized human keratinocytes (HPK1A), but this inhibition was less in the more malignant ras-transfected clone HPK1A-ras [168,169]. 1,25-$(OH)_2D_3$ and the analogs EB1089 and 22-oxacalcitriol inhibit the PTHrP gene transcription in and release from the squamous cancer cell line NCI H520 [170]. In addition, in the human T-cell lymphotrophic virus type I (HTLV-I)-transfected T-cell line MT-2, 1,25-$(OH)_2D_3$ and 22-oxacalcitriol did inhibit PTHrP gene expression and PTHrP secretion [171]. In rat H-500 Leydig tumor cells [172], and PC-3 prostate cancer cells 1,25-$(OH)_2D_3$ inhibited PTHrP secretion. It was suggested that this might play a role in the growth inhibition by vitamin D as PTHrP stimulates prostate cancer growth, tumor invasion, and metastasis [173–175]. *In vivo* observations comparable to these *in vitro* observations have also been made. When these H-500 Leydig tumor cells were implanted in Fisher rats, treatment with 1,25-$(OH)_2D_3$ and the analog EB1089 resulted in reduced levels of tumor PTHrP mRNA and PTHrP serum levels [97]. EB1089 also reduced serum levels of PTHrP in nude mice implanted with squamous cancer cells [176]. In Fisher rats implanted with the Walker carcinoma, 1,25-$(OH)_2D_3$ caused a decrease in serum PTHrP, but the ratio of PTHrP levels and tumor weight was similar in rats receiving vehicle or 1,25-$(OH)_2D_3$. The data point to an indirect effect on PTHrP via growth inhibition. However, the PTHrP mRNA levels appeared to be decreased by 1,25-$(OH)_2D_3$ [100]. In nude mice bearing the FA-6 cell line of a pancreas carcinoma lymph node metastasis, 22-oxacalcitriol inhibits PTHrP gene expression, which is related to inhibition of

tumor-induced hypercalcemia [177]. Together, the overall picture that emerges from these studies is that an important additional anticancer effect of vitamin D_3 and analogs could be the inhibition of the humoral hypercalcemia of malignancy.

In contrast to these inhibitory effects in human tumor cells and tumor models, a stimulatory effect of 1,25-$(OH)_2D_3$ and EB1089 on PTHrP gene transcription and PTHrP production by a canine oral squamous carcinoma cell line (Sec 2/88) has been observed [178,179]. Also in an *in vivo* model of canine adenocarcinoma CAC-8 implanted in nude mice, stimulation of PTHrP by 1,25-$(OH)_2D_3$ and EB1089 was observed [179]. These data indicate that the effect of vitamin D and analogs on canine tumors differs from that on human tumors.

III. VITAMIN D EFFECTS ON TUMOR CELLS

A. Cell Cycle

It has now been well established that vitamin D inhibits growth of cells by interfering with the cell cycle. Proliferating cells progress through the cell cycle, which comprises the G_0/G_1 phase (most differentiated, nondividing cells are in the G_1 phase), the S phase in which new DNA is synthesized, and the G_2 phase, which is followed by mitosis (M phase) whereon the cells reenter the G_0/G_1 phase. In most of the cells studied so far, treatment with 1,25-$(OH)_2D_3$ and its analogs results in a blockade at a specific checkpoint, i.e., the restriction point (R), in the G_1 phase limiting the transition of G_1 to S and reducing the number of cells in S phase. Some studies also have examined the effect on the G_2 phase, but these results are somewhat more diverse. In general it can be concluded that blocking the transition from the G_0/G_1 phase to the S phase plays an important role in the growth inhibitory effect of 1,25-$(OH)_2D_3$.

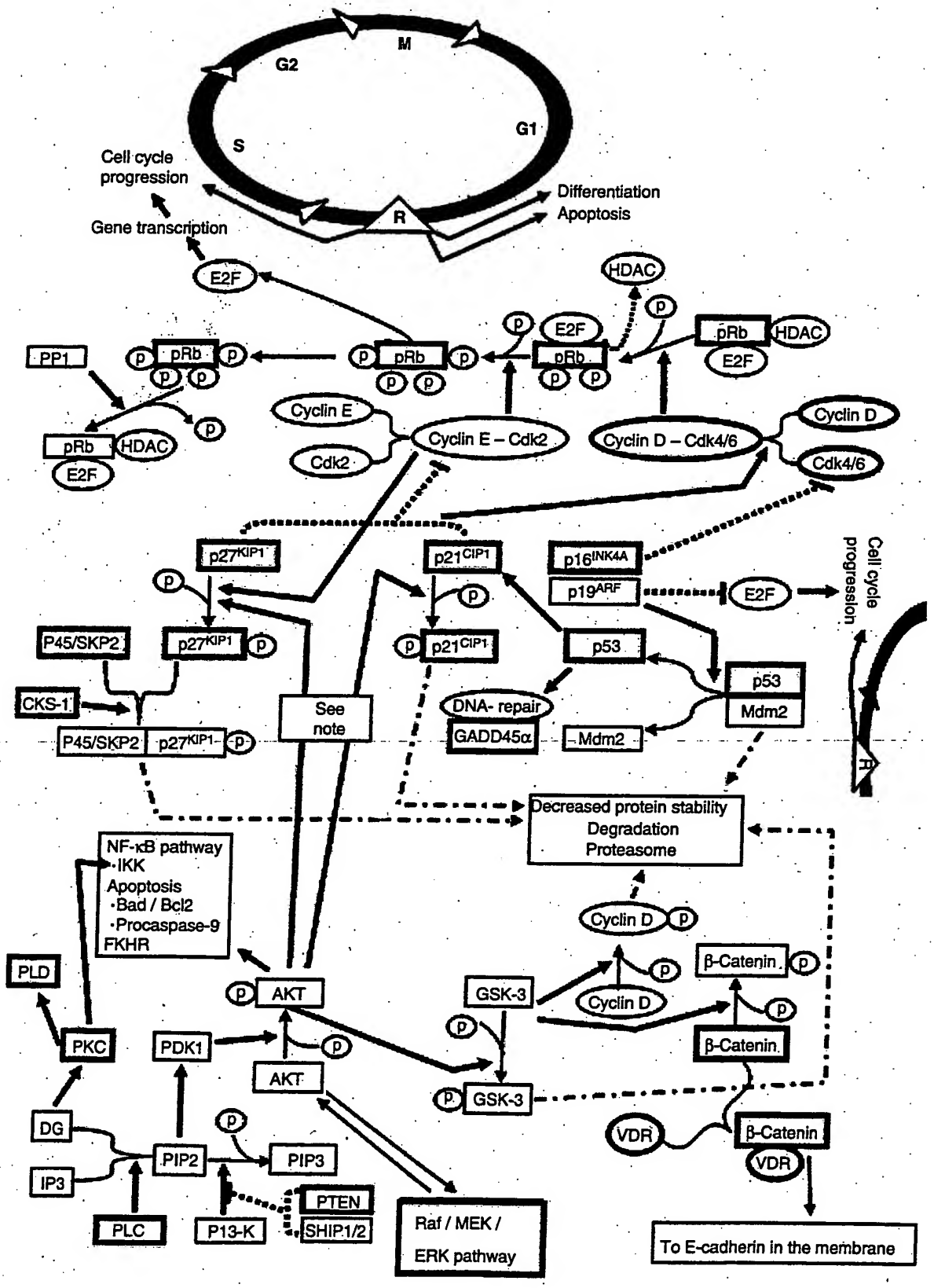
In the regulation of the cell cycle, numerous genes and proteins have been described. It is beyond the scope of this chapter to discuss in detail the regulation of all of the genes/proteins by vitamin D. In Fig. 1, an overview is given of the interacting genes/proteins that are involved in intracellular signaling and regulating the cell cycle. These genes and proteins are part of the cascade of events on which vitamin D exerts its effects. The components shown to be regulated by vitamin D are indicated. Figure 1 is a compilation of data present so far; it is important to realize that probably not all genes/proteins are affected by vitamin D in all tumor cells. However, in this way one gets an overview of the broad range of effects of vitamin D on intracellular signaling pathways involved in regulation of (tumor) cell growth. More details on the regulation of the cell cycle will be discussed in several other chapters, especially Chapter 92.

Besides its effects on cell cycle regulation, vitamin D has recently been implicated to be involved in control of genomic stability [180]. 1,25-$(OH)_2D_3$ has been reported to inhibit hepatic chromosomal aberrations and DNA strand breaks [181]. This is supported by the finding that 1,25-$(OH)_2D_3$ and EB1089 stimulated the expression of GADD45, which stimulates DNA repair [182] and might be coupled to release of p53 from Mdm2 (see Fig. 1).

1. (ONCO)GENES AND TUMOR SUPPRESSOR GENES

Oncogenes and tumor suppressor genes generally are involved in control of the cell cycle and apoptosis (see Chapter 92). One of the most widely studied oncogenes in relation to vitamin D is *c-myc*. C-Myc suppresses expression of cell cycle/growth arrest genes gas1, p15, p21, p27, and gadd34, -45, and -153 [183]. C-Myc has been postulated to play an early role in the following cascade of events in G_1: cyclins activate cyclin-dependent kinases (CDKs), which in turn can phosphorylate the retinoblastoma tumor suppressor gene product ($p110^{RB}$), resulting in transition from G_1 to S phase (see Fig. 1). In HL-60 cells, breast cancer cells, and several other cell types, 1,25-$(OH)_2D_3$ has been reported to decrease *c-myc* oncogene expression [184–189]. Analysis of HL-60 sublines showed a relation between reduction of c-*myc* expression and inhibition of proliferation [190]. Similar observations were made for neuroblastoma cells treated with 1,25-$(OH)_2D_3$, EB1089, and KH10560 [191]. We did not observe a 1,25-$(OH)_2D_3$-induced change in *c-myc* expression in MCF-7 and ZR-75.1 breast cancer cells while they were both growth inhibited [192], and a similar observation has been made for the colon-adenocarcinoma CaCo-2 cell line [193].

Nontransformed embryonic fibroblasts are growth inhibited by 1,25-$(OH)_2D_3$, whereas *c-myc* is not changed or is even increased [194,195]. In the MG-63 osteosarcoma cell line, 1,25-$(OH)_2D_3$ has been shown to enhance *c-myc* expression [196], whereas we observed growth inhibition by 1,25-$(OH)_2D_3$ [197]. These data show that regulation of c-myc expression may be part of growth inhibition by vitamin D, but that this is not generally applicable to all cells. 1,25-$(OH)_2D_3$ has also been reported to regulate expression of other oncogenes, like *c-myb*, *c-fos*, *c-fms*, *c-fra1*, *c-jun*, *junD*, *c-Ki-ras*, *N-ras*, *c-src* [189,198–203]; however, these data are rather limited. Nevertheless, it is clear that 1,25-$(OH)_2D_3$ has effects on the expression of various oncogenes. The data so far are not conclusive with respect to which genes are crucial in the growth inhibitory action of 1,25-$(OH)_2D_3$. This can be attributed to the fact that these (proto)oncogenes encode for transcription factors,


M
G2
G1
S
R
Cell cycle progression
Gene transcription
Differentiation
Apoptosis
E2F
HDAC
PP1
pRb
p
Cyclin E
Cdk2
Cyclin E – Cdk2
Cyclin D – Cdk4/6
Cyclin D
Cdk4/6
p27KIP1
p21CIP1
p16INK4A
p19ARF
E2F
Cell cycle progression
P45/SKP2
CKS-1
p53
Mdm2
DNA- repair
GADD45α
See note
Decreased protein stability
Degradation
Proteasome
NF-κB pathway
·IKK
Apoptosis
·Bad / Bcl2
·Procaspase-9
FKHR
PLD
PKC
PDK1
AKT
GSK-3
β-Catenin
Cyclin D
DG
IP3
PIP2
PIP3
PLC
P13-K
PTEN
SHIP1/2
Raf / MEK / ERK pathway
VDR
To E-cadherin in the membrane

growth factor receptors, or components or intracellular signaling cascades. The effects of these may differ between cells dependent on presence or absence of additional cell type specific conditions. Therefore, their postulated role is often complex. For example, increased *c-myc* expression can be related to induction of apoptosis but also to stimulation of cell cycle progression.

In contrast to the oncogenes, the effect of 1,25-$(OH)_2D_3$ on the retinoblastoma tumor suppressor gene is much clearer. This may be related to the fact that, in contrast to oncogenes, retinoblastoma and p53 take well-defined positions in the control of cell cycle and DNA repair (see Fig. 1). The $p110^{RB}$ retinoblastoma gene product can either be phosphorylated or dephosphorylated. In the phosphorylated form, it can activate several transcription factors and cause transition to S phase and DNA synthesis. In human chronic myelogenous leukemia cells [204], breast cancer cells [205], and HL-60 cells [206,207], 1,25-$(OH)_2D_3$ caused a dephosphorylation of $p110^{RB}$, which is related to growth inhibition and cell cycle arrest in G_0/G_1 and also in G_2 [207]. In leukemic cells, 1,25-$(OH)_2D_3$ also caused a reduction in the cellular level of $p110^{RB}$ [204,206]. In nontransformed keratinocytes, 1,25-$(OH)_2D_3$ induced dephosphorylation of $p110^{RB}$ as well [208]. The other major tumor suppressor gene is p53. For leukemic U937 cells, it was reported that presence of p53 is important for 1,25-$(OH)_2D_3$-induced differentiation [209]. In rat glioma cells, 1,25-$(OH)_2D_3$ induces expression of p53 [210]. However, 1,25-$(OH)_2D_3$ can inhibit cell growth and induce differentiation in cancer cells with defective p53 [211] and also p53-independent induction of apoptosis by EB1089 has been demonstrated [212]. These latter observations might be explained by the fact that vitamin D also interferes at levels in the cascade of cell cycle control down-stream of p53 (see Fig. 1). Recently, an additional interesting relationship between tumor suppressor genes and vitamin D has recently been shown for the Wilms' tumor suppressor gene WT1. This zinc-finger containing transcription factor induces transcription of the VDR gene [213].

Several interesting additional genes and vitamin D targets in cancer treatment should be mentioned. First in 1994 Chen and DeLuca isolated and characterized a vitamin D–induced gene in HL-60 cells [214]. This protein, vitamin D-up-regulated protein (VDUP1), is a thioredoxin-binding protein-2 [215]. Thioredoxin has several roles in processes such as proliferation or apoptosis. It also promotes DNA binding of transcription factors such as NF-κB, AP-1, p53, and PEBP2. In addition, overexpression of thioredoxin suppresses the degradation of IκB and the transactivation of NF-κB, whereas overexpression of nuclear-targeted thioredoxin exhibits the enhancement of NF-κB-dependent transactivation [216]. However, it is only in more recent studies that a relationship between VDUP1 and cancer has been established. The expression of VDUP1 was found to correlate with malignant status of colorectal and gastric cancers [217]. 5-fluorouracil, which is widely used for treatment of colon cancer, induces VDUP1 expression in the SW620 colon cancer cell line [218]. In smooth muscle cells and cardiomyocytes VDUP1 inhibits proliferation and is involved in induction of apoptosis [219,220]. An association with vitamin D effects on cancer is made by two recent studies showing induction of VDUP1 by 1,25-$(OH)_2D_3$ in tumor cells and that VDUP1 induces cell cycle arrest [221,222]. Moreover, interaction with histone deacetylase (HDAC; see Fig. 1), promyelocytic leukemia zinc-finger (PLZF) was demonstrated. Interestingly and further complicating the story, PLZF inhibits 1,25-$(OH)_2D_3$ induced differentiation of U937 leukemic cells by binding to the VDR and inhibiting gene transcription [223,224]. Interestingly, the gene, DRH1, was cloned from hepatocellular carcinoma, and its expression was strongly reduced in cancer tissue compared to normal liver [225]. DRH1 has a 41% homology with VDUP1. Whether this points to a new family of cancer genes remains to be established, but it certainly opens new venues for intervening in cancer cell growth.

FIGURE 1 Schematic representation summarizing the intracellular pathways and signaling pathways involved regulation of the cell cycle shown to be regulated by 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs in regulating cell proliferation. Targets shown to be affected by 1,25-$(OH)_2D_3$ and/or its analogs are indicated in the bold boxes and ovals. Bold arrows and fine dotted lines indicate stimulation and inhibition, respectively. Coarse dotted lines indicate processing to the proteasome. p indicates phosphorylation. The effects on these cellular targets are not demonstrated in all types of cancer cells but this diagram is aimed to give an overview of demonstrated targets and potential targets. NOTE: Dependent on the site of phosphorylation proteins can either be destabilized or degraded or be stabilized and activated. For example: phosphorylation of p21 at T145 by AKT leads to degradation while phosphorylation of S146 by AKT leads to increased stability.
Abbreviation used: AKT (PKB), Protein kinase B; Bad, BCL2-antagonist of cell death; Bcl2, B-cell leukemia/lymphoma 2; Cdk, Cyclin-dependent kinase; CKS-1, Cyclin kinase subunit 1; DG, Diacylglycerol; E2F, Transcription factor; ERK, Extracellular-signal regulated kinase; FHKR (AFX/FOX), Forkhead family of transcription factors; GSK-3, Glycogen synthase kinase-3; HDAC, Histone deacetylase; IKK, I-κB kinase; IP3, Inositol 1,4,5-trisphosphate; Mdm2, Mouse double minute 2; MEK, Raf-1-MAPK/ERK kinase; PDK1, Phosphatidylinositol-dependent kinase 1; PI3-K, Phosphatidyl inositol 3 kinase; PIP2, Phosphatidylinositol (4,5)-phosphate; PIP3, Phosphatidylinositol (3,4,5) phosphate; PKC, Protein kinase C; PLC, Phospholipase C; PLD, Phospholipase D; PP!, Protein phosphatase 1-like protein; pRB, Retinoblastoma protein; PTEN, Phosphatase and tensin homologue; SHIP 1 and 2, Src homology 2 (SH2) containing phosphatases 1 and 2; SKP2, Ubiquitin ligase; VDR, Vitamin D receptor.

Second, an additional therapeutic target for vitamin D compounds might be regulation of enzymes involved in estrogen and androgen synthesis and metabolism [226–229]. Third, telomerase activity provides a mechanism for unlimited cell division. In HL-60 cells, 1,25-$(OH)_2D_3$ inhibits telomerase activity [230]. Fourth, the homeobox genes may prove to be a major target for vitamin D action in cancer, but this possibility remains to be elucidated. In a differential expression screen using the human U937 leukemic cells, the HoxA10 gene was shown to be regulated by 1,25-$(OH)_2D_3$ [231].

It is to be expected that as a result of the increasing application of large scale microarray gene expression analyses, a vast number of new cell cycle and vitamin D regulated genes will be identified and add to the unraveling and understanding of vitamin D control of cancer cell proliferation [232–235].

B. Apoptosis

A block in the cell cycle preventing transition into S phase may cause cells to go either into apoptosis or to enter a specific differentiation pathway (see Chapter 93). What exactly determines the decision of apoptosis or differentiation remains to be elucidated. It is suggested that early G_1 phase may be the point at which switching between cell cycle progression and induction of apoptosis occurs [236,237].

Induction of apoptosis, an orderly and characteristic sequence of biochemical, molecular, and structural changes resulting in the death of the cell [238], is a mechanism by which 1,25-$(OH)_2D_3$ inhibits tumor cell growth and may contribute to tumor suppression and explain the reduction in tumor volume found in various *in vivo* animal studies (see Section II.C).

1,25-$(OH)_2D_3$ has been shown to regulate expression of apoptosis genes and to induce apoptosis of cancer cells of various origins. For example, 1,25-$(OH)_2D_3$ and the analog Ro 25-6760 cause a cell cycle block in HT-29 human colon cancer cells, resulting in growth inhibition and induction of apoptosis [239]. The *bcl-2* oncogene decreases the rate of programmed cell death [240,241]. However, protection of HL-60 cells against apoptosis occurred despite down-regulation of *bcl-2* gene expression [242]. In several breast cancer cell lines (MCF-7, BT-474, MDA-MB-231) 1,25-$(OH)_2D_3$ and the analogs KH1060 and EB1089 decreased *bcl-2* expression [211,243]. The analog CB1093 reduced *bcl-2* expression in MCF-7 cells associated with the induction of apoptosis [244]. However, only in MCF-7 cells has this change in *bcl-2* expression been accompanied by apoptosis. Effects on other genes/proteins have also been reported [245], and microarray gene expression analyses and differential screening will also definitively reveal additional vitamin D targets in regulating apoptosis [246].

A central role for apoptosis in the action of 1,25-$(OH)_2D_3$ is unclear because growth inhibition of several other breast cancer cells appeared to be independent of apoptosis [211]. Also, MCF-7 cells that showed growth inhibition by 1,25-$(OH)_2D_3$ could, after removal of the hormone, again be stimulated to grow, implying transient growth inhibition and not cell death [247]. Stable transfection of leukemic U937 cells with the wild-type p53 tumor suppressor gene resulted in a reduced growth rate and produced cells that can undergo either apoptosis or maturation. In these cells 1,25-$(OH)_2D_3$ protects against p53-induced apoptosis and enhances p53-induced maturation [209]. In two independent studies with HL-60 cells, 1,25-$(OH)_2D_3$ was found either to protect against or to have no effects on apoptosis [242,248]. Vitamin D protection against apoptosis was also detected in human U937 leukemic cells treated tumor necrosis factor α [249]. Absence of a vitamin D effect on apoptosis might be explained by the expression of the antiapoptotic protein BAG-1 p50 isoform. This protein has been shown to bind to the VDR and block vitamin D–induced transcription [250]. The presence of additional interacting factors might also be important for the eventual effect on apoptosis as in the study with HL-60 cells, which in the presence but not the absence of 9-cis-retinoic acid, 1,25-$(OH)_2D_3$ did induce apoptosis [248]. The role of vitamin D interaction with other factors will be discussed in more detail in Section IV. In summary, the data obtained so far show that 1,25-$(OH)_2D_3$-induced growth inhibition can be related to apoptosis in some cases, but that growth inhibition is frequently observed to be independent of apoptosis. Possibly in these latter cases, induction of differentiation is more prominent. The factor that decides whether cells undergo apoptosis or differentiation is unclear but is probably dependent on cell cycle stage, presence of other factors, and levels of expression of oncogenes and tumor suppressor genes. An interesting phenomenom to be studied concerning vitamin D and apoptosis is calbindin 28K. Calbindin 28K is a well-known vitamin D–induced protein that has recently been shown to inhibit apoptosis [251]. It is tempting to speculate that calbindin 28K plays a role in the decision whether vitamin D induces cells to differentiate or to go into apoptosis or that it is involved when 1,25-$(OH)_2D_3$ protects against apoptosis (see Chapter 42).

C. Differentiation

In addition to proliferation and apoptosis, the third major cellular process is differentiation. As described

above for the classic actions of 1,25-$(OH)_2D_3$ related to calcium homeostasis, effects on cell differentiation and proliferation are involved. The coupling between proliferation and differentiation has been most widely studied for cells of the hematopoietic system (Chapter 96) and keratinocytes (Chapter 35). In general, 1,25-$(OH)_2D_3$ inhibits proliferation and induces differentiation along the monocyte-macrophage lineage. Rapidly proliferating and poorly differentiated keratinocytes can be induced to differentiate by 1,25-$(OH)_2D_3$. A further relationship between the vitamin D_3 system and differentiation is demonstrated by the fact that in poorly differentiated keratinocytes 1,25-$(OH)_2D_3$ production and vitamin D receptor levels are high, whereas after induction of differentiation these levels decrease [252], and in melanoma cells 1,25-$(OH)_2D_3$ stimulates melanin production [253]. Effects on differentiation have also been reported for other cell types. Inhibition of prostate cancer cell proliferation is paralleled by an increased production of prostate specific antigen [254–257]. In the BT-20 breast cancer cells 1,25-$(OH)_2D_3$ induced morphological changes indicative for differentiation [258]. In several breast cancer cell lines, the stimulation of differentiation has been established by determining lipid production by the cells [211]. In this study, Elstner *et al.* demonstrated an uncoupling between effects on proliferation and differentiation. In two breast cancer cell lines, 1,25-$(OH)_2D_3$ and various analogs induced differentiation even though the cells were resistant to cell cycle and antiproliferative effects. This finding, together with data obtained with human myelogenous leukemia cells, [204] suggests a dissociation between the cellular vitamin D_3 pathways involved in regulation of differentiation and proliferation (see also Section V). For a HL-60 subclone, a similar observation was made [190], and in another HL-60 subclone the induction of differentiation was found to precede the G_0/G_1 cell cycle block. In contrast to the above-mentioned observations on stimulation of differentiation, 1,25-$(OH)_2D_3$ inhibits erythroid differentiation of the erythroleukemia cell line K562 [186], and 1,25-$(OH)_2D_3$ inhibits Activin A-induced differentiation of murine erythroleukemic F5-5 cells [259]. Although precise relationships among growth inhibition, cell cycle effects, and apoptosis are unclear, it can be concluded that an important effect of vitamin D_3 on both normal and malignant cells is induction of differentiation.

D. Growth Factors and Growth Factor Receptors

Besides regulation of cell cycle-related oncogenes and tumor suppressor genes, interaction with tumor- or stroma-derived growth factors is important for growth inhibition. Stimulation of breast cancer cell proliferation by coculture with fibroblasts is inhibited by 1,25-$(OH)_2D_3$ [260]. A good candidate to interact with the 1,25-$(OH)_2D_3$ action is transforming growth factor-β (TGFβ). TGFβ is involved in cell cycle control and apoptosis [261,262]. TGFβ can interfere with the cascade of events in the GI phase described above and inhibit the ability of cells to enter S phase when the factor is present during the GI phase. TGFβ has been shown to suppress *c-myc*, cyclin A, cyclin E, and cdk2 and cdk4 expression [262]. In line with this, TGFβ has been reported to inhibit phosphorylation of $p110^{RB}$ [263]. Vitamin D_3 compounds induce dephosphorylation of the retinoblastoma gene product, and vitamin D_3 growth inhibition of MCF-7 breast cancer cells is inhibited by a TGFβ neutralizing antibody [264]. 1,25-$(OH)_2D_3$ and several analogs stimulated the expression of TGFβ mRNA and secretion of active and latent $TGF\beta_1$ by the breast cancer cell line BT-20 [154]. 1,25-$(OH)_2D_3$ enhanced $TG\beta_1$ gene expression in human keratinocytes [265] and the secretion of TGFβ in murine keratinocytes [266]. In both studies, antibodies against TGFβ inhibited the growth inhibitory effect of vitamin D_3. Further evidence for a vitamin D_3-TGFβ interaction is that bone matrix of vitamin D–deficient rats contains substantially less TGFβ than controls [267]. Therefore, on the basis of these consistent findings, TGFβ is a likely candidate to play a role in the 1,25-$(OH)_2D_3$-induced growth inhibition [268].

Interactions with the insulin-like growth factor (IGF) system have also been described. IGFs are potent growth stimulators of various cells, and their effect is regulated via a series of IGF binding proteins (IGFBPs). 1,25-$(OH)_2D_3$ and the analog EB1089 inhibit the IGF-I-stimulated growth of MCF-7 breast cancer cells [269]. In prostate cancer cell lines, 1,25-$(OH)_2D_3$ induced expression of IGFBP6 but not IGFBP4 [270]. In human osteosarcoma cell lines, 1,25-$(OH)_2D_3$ and the analog 1α-dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol potently stimulated the expression and secretion of IGFBP3 [271–273]. In one study an association has been made between increased IGFBP3 levels and 1,25-$(OH)_2D_3$ growth inhibition [271]. Recent observations that antisense oligonucleotides to IGFBP3 prevented growth inhibition of prostate cancer cells by 1,25-$(OH)_2D_3$ [235] provided further evidence for an interplay between 1,25-$(OH)_2D_3$ and IGFBP3. Interestingly, in the human osteosarcoma cell line MG-63, 1,25-$(OH)_2D_3$ and TGFβ synergistically increased IGF-BP-3 secretion [273]. An example of growth factor receptor regulation by 1,25-$(OH)_2D_3$ concerns the epidermal growth factor (EGF) receptor. This receptor is down-regulated in T47-D breast cancer cells and up-regulated in BT-20 breast cancer cells. Nevertheless, 1,25-$(OH)_2D_3$ inhibits the growth of both

cell lines [274,275]. These data provide evidence that interactions with growth factors are only part of the 1,25-$(OH)_2D_3$ action on tumor cells.

As described above, it is clear that 1,25-$(OH)_2D_3$ has effects on the expression of various oncogenes and tumor suppressor genes and that multiple interactions with various growth factors exist. However, the data on these aspects, separately as well as in combination, are still too limited to define a distinct mechanism of action for the 1,25-$(OH)_2D_3$ anticancer effects. However, with respect to growth inhibition, at this time two models of action can be postulated. In the first one, 1,25-$(OH)_2D_3$ directly interferes with a crucial gene(s) involved in the control of the cell cycle. In this case, in view of the general pattern of the genes involved in cell cycle control, this mechanism of action will be similar in all types of cancer cells. However, the effect on cell cycle genes will be dependent on the presence or absence of additional growth factors. This will determine, depending on which growth factors are present, the differences in 1,25-$(OH)_2D_3$ action between cancer types of different origin but also within cancer types of similar origin. The second model is based on an indirect effect of 1,25-$(OH)_2D_3$ on cell cycle progression and tumor growth. In this case 1,25-$(OH)_2D_3$ may either inhibit or potentiate the effect of growth stimulatory or inhibitory factors, respectively, via, for example, effects on growth factor production, growth factor binding protein levels, or receptor regulation. It is also conceivable that a combination of both models forms the basis of 1,25-$(OH)_2D_3$ regulation of tumor cell growth.

IV. COMBINATION THERAPY

The data obtained with 1,25-$(OH)_2D_3$ and its analogs on growth inhibition and stimulation of differentiation offer promise for their use as an endocrine anticancer treatment. Single agent treatment with low calcemic 1,25-$(OH)_2D_3$ analogs could be useful; however, combination therapy with other tumor effective drugs may provide an even more beneficial effect. Up to now several *in vitro* and *in vivo* studies have focused on possible future combination therapies with 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs.

For breast cancer cells the combination of the presently most widely-used endocrine therapy, the antiestrogen tamoxifen, with 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs resulted in a greater growth inhibition of MCF-7 and ZR-75-1 cells than treatment with either compound alone [118,192,247]. In combination with tamoxifen, the cells were more sensitive to the antiproliferative action of 1,25$(OH)_2D_3$ and the analogs; that is, the EC_{50} values of the vitamin D_3 compounds in the presence of tamoxifen were lower than those in the absence of tamoxifen. Studies with MCF-7 cells suggested a synergistic effect of 1,25$(OH)_2D_3$ and tamoxifen on apoptosis [276]. In addition, in *in vivo* breast cancer models a synergistic effect of the tamoxifen-1,25$(OH)_2D_3$ analogs combination was observed [118,119]. Additional data on the interaction between the estrogen/antiestrogen system and vitamin D comes from studies showing the presence of an estrogen responsive element in the VDR promoter and regulation of VDR by estradiol in breast cancer cells [277]. This is intriguing that the stimulator of breast cancer cell growth induces the expression of the receptor for a growth inhibitor. VDR up-regulation in breast cancer cells and increased transcriptional activity was mimicked by the phytoestrogens resveratrol and genistein and blocked by tamoxifen [278]. In colon cancer also, VDR up-regulation by estradiol has been reported. However, in colon it was hypothesized to contribute to the protective effect of estradiol on chemically-induced colon carcinogenesis [279].

These important and complex interactions between the vitamin D and estrogen endocrine system in the regulation of cancer (cells) are promising and warrant further detailed analyses, e.g. regarding tissue(cancer)-specific effects. In addition, the estrogen endocrine system may regulate the metabolism of 1,25-$(OH)_2D_3$ in cancer cells and thereby affect its action (see Section V). Interaction with another sex steroid, testosterone, has been described for ovarian cancer. Vitamin D inhibits dihydrotestosterone (DHT) and DHT stimulation of ovarian cancer cells [280]. Intriguingly, also here the growth stimulator and growth inhibitor mutually up-regulate each others receptors. Also, in prostate cancer cells, it has been shown that 1,25-$(OH)_2D_3$, while inhibiting androgen stimulated growth, up-regulates the androgen receptor [281].

Interaction with another steroid in regulating cancer cells had already been reported in 1983. The synthetic glucocorticoid, dexamethasone, and 1,25-$(OH)_2D_3$ synergistically induced differentiation of murine myeloid leukemia cells [282]. This was supported by *in vitro* and *in vivo* data showing that dexamethasone enhanced the effect of vitamin D on growth inhibition, cell cycle arrest, and apoptosis of squamous carcinoma cells [283,284]. A possible mechanism is the up-regulation of VDR by dexamethasone [283]. An interesting aspect of this combination is not only the direct interaction at cancer cell level, but also in the control of the calcemic action of 1,25-$(OH)_2D_3$. Glucocorticoids inhibit intestinal calcium absorption and increase renal calcium excretion and in this way it may limit the hypercalcemic action of 1,25-$(OH)_2D_3$ [285].

Combination of vitamin D_3 and retinoids has been examined in various systems. A combination of retinoic acid and 1,25-$(OH)_2D_3$ resulted in a more profound inhibition of both T47-D breast cancer cells [286] and LA-N-5 human neuroblastoma cells [287]. 9-cis-Retinoic acid augmented 1,25-$(OH)_2D_3$-induced growth inhibition and differentiation of HL-60 cells [288]. Besides growth inhibition and differentiation effects, the combination of 1,25-$(OH)_2D_3$ and various isomers of retinoic acid were more potent in reducing angiogenesis than either compound alone [146–148]. The background of the interaction between retinoids and 1,25-$(OH)_2D_3$ may be attributed to heterodimer formation of the respective receptors [289].

For several cytokines, interactions with 1,25-$(OH)_2D_3$ have been described. Interferon-γ and 1,25-$(OH)_2D_3$ synergistically inhibited the proliferation and stimulated the differentiation of HL-60, WEHI-3, and U937 myeloid leukemia cells [290–293]. Treatment of LLC-LN7 tumor cells with 1,25-$(OH)_2D_3$ with IFN-γ synergistically reduced tumor granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion and a blockage in the capacity of the tumor cells to induce granulocyte-macrophage-suppressor cells [99]. In the mouse myeloid leukemia cell line Ml interleukin-4 enhanced 1,25$(OH)_2$ D_3-induced differentiation [189,294,295]. Also with interleukin-1β, interleukin-3, interleukin-6, and interleukin-12 interactions with 1,25-$(OH)_2D_3$ have been reported [296–298]. 1,25-$(OH)_2D_3$ and tumor necrosis factor synergistically induced growth inhibition and differentiation of HL-60 [299]. For MCF-7 cells an interaction between 1,25-$(OH)_2D_3$ and tumor necrosis factor has also been reported [298,300]. In the presence of GM-CSF, lower concentrations of 1,25$(OH)_2D_3$ could be used to achieve a similar antiproliferative effect in MCF-7 cells [301] and to induce differentiation of U937 myeloid leukemic cells [302]. Other factors shown to interact with 1,25-$(OH)_2D_3$ are butyrate [303–305], melatonin [306], EGF [307], and the factors described in Section III.C.

Furthermore, combinations of vitamin D_3 compounds with cytotoxic drugs, antioxidants, and radiation have been studied. *In vivo* adriamycin and *in vitro* carboplatin, cisplatin, and doxorubicin interacted synergistically with 1,25-$(OH)_2D_3$ to inhibit breast cancer cell growth [113,308–311]. In a carcinogen-induced rat mammary tumor model, treatment with 1α-$(OH)D_3$ and 5-fluorouracil, however, did not result in enhanced antitumor effects [96]. Recently, interactions with a plant-derived polyphenolic antioxidant, carnosic acid were demonstrated in the differentiation of HL-60 cells, which was related to a decrease in the intracellular levels of reactive oxygen species [312,313]. Also interaction with radiation therapy in breast cancer has been described [314–316].

The data on combinations of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs with various other anticancer compounds are promising and merit further analyses. The development of effective combination therapies may result in better response rates and lower required dosages, thereby reducing the risk of negative side effects.

V. RESISTANCE AND VITAMIN D METABOLISM

Classic vitamin D resistance concerns the disease hereditary vitamin D–resistant rickets, which is characterized by the presence of a nonfunctional VDR and consequently aberrations in calcium and bone metabolism (see Chapter 72). For cancer cells, the presence of a functional VDR is also a prerequisite for a growth regulatory response, and a relationship between VDR level and growth inhibition has been suggested for osteosarcoma, colon carcinoma, breast cancer, prostate cancer cells, and rat glioma [1,2,108,129,205,210,317–321]. Cell lines established from DMBA-induced breast tumors in VDR knockout mice are insensitive to growth arrest and apoptosis by 1,25-$(OH)_2D_3$, EB1089 and CB1093 [322]. Albeit that VDR is a prerequisite for tumor cell growth regulation, the presence of the VDR is not always coupled to a growth inhibitory response of 1,25$(OH)_2D_3$. Results from studies with transformed fibroblasts [194], myelogenous leukemia cells [190,204,323], transformed keratinocytes [187], and various breast cancer cell lines [211,324] demonstrated a lack of growth inhibition by 1,25$(OH)_2D_3$ even in the presence of VDR. In this situation, the designation "resistant" is based on the lack of growth inhibition, even though, as discussed earlier in Section III.C, some of these cells are still capable of being induced to differentiate [204,211]. This points to a specific defect in the growth inhibitory pathway. In the resistant MCF-7 cells, this defect is not located at a very common site in the growth inhibitory pathway of the cell because the growth could still be inhibited with the antiestrogen tamoxifen [324]. For myelogenous leukemia cells, similar observations have been made [325].

For VDR-independent resistance to growth inhibition, the underlying mechanism(s) is unknown. For the resistant MCF-7 clone, this is not related to up-regulation of the P-glycoprotein [324]. Interestingly, these vitamin D–resistant MCF-7 clones can be sensitized to 1,25$(OH)_2$D by activation of protein kinase C, resulting in induction of apoptosis and transcriptional activation, suggesting that alterations in phosphorylation may affect vitamin D sensitivity [326]. An interesting growth

inhibition resistant MCF-7 cell clone was described by Hansen *et al.* This clone was not growth inhibited while VDR was still present and 24-hydroxylase could still be induced [327]. Other examples of vitamin D resistance are HL60 cells that have been cultured for four years in the presence of 1,25-$(OH)_2D_3$ and resulted in clones that are resistant to differentiation inducing and growth inhibition. They became not only resistant to 1,25$(OH)_2$D but also to 5-beta-D-arabinocytosine, suggesting a common metabolic pathway being responsible [328]. Whether this relates to the up-regulation of the multidrug resistance proteins is not clear. In the resistant leukemia $JMRD_3$ cell line, altered regulation and DNA–binding activity of *junD* as part of the AP-1 complex has been reported [200]. Resistance to growth inhibition in the presence of VDR has also been linked to disruption of the VDR-RXR complex [329] and increased RXR degradation [330]. In addition, other factors, like the acute myeloid leukemia translocation products (e.g. PLZF) may contribute to resistance to vitamin D by sequestering the VDR [223,224].

The 1,25$(OH)_2D_3$ sensitive and resistant cell clones provide interesting models to examine the molecular mechanisms of 1,25$(OH)_2D_3$-induced growth inhibition. For example, lack of p21 results in no cell cycle block [331] and no apoptosis was detected with a mutated p53 [211]. Finally, the recent identification of cellular proteins that are involved in the vitamin D resistance in new world primates might add to the understanding of tumor cell resistance to vitamin D [332,333] (see Chapter 21).

At this time, the major mechanism for vitamin D resistance or reduced sensitivity in VDR containing tumor and cancer cells is 1,25-$(OH)_2D_3$ catabolism via the C24-hydroxylation pathway. An inverse relationship between cellular metabolism of 1,25-$(OH)_2D_3$ via 24-hydroxylation and growth inhibition of prostate cancer cells has been suggested [318]. The latter observation is intriguing, the more so as an inverse relationship between VDR level and induction of 24-hydroxylase (CYP24) activity was reported. In general, there may exist a direct relationship between VDR level and induction of 24-hydroxylase activity [319,334]. An important role in the control of 1,25-$(OH)_2D_3$ action on cancer cells was provided by studies with the 1,25-$(OH)_2D_3$–resistant prostate cancer cell line DU145. It was shown that 1,25-$(OH)_2D_3$ did inhibit the growth of these cells when it was combined with the 24-hydroxylase inhibitor Liazorole [335]. Inhibition of 24-hydroxylase activity in HL-60 cells also altered the effect of 1,25-$(OH)_2D_3$ and 20-epi analogs [336]. The action of the analog EB1089 was also limited by hydroxylation at the C24 position [337]. However, it was suggested that the increased potency of EB1089 is at least partly due to resistance to 24-hydroxylation [234]. Alternatively, 24-hydroxylation of the analog KH1060 has been implicated as one of the mechanisms to explain the potency of this analog. The 24-hydroxylated metabolites of this analog are very stable and are biologically active [338,339]. It has been shown that the naturally occurring 24-hydroxylated metabolite of vitamin D_3, 24R,25-$(OH)_2D_3$, also has a preventive effect on chemically-induced colon cancer [340].

Interaction between the estrogen system and 24-hydroxylase is also of importance. Recent data have shown that the phytoestrogen genistein inhibits 24-hydroxylase activity in prostate cancer cells and thereby increases the responsiveness to 1,25-$(OH)_2D_3$ [341]. A role for 24-hydroxylase as oncogene is suggested by data showing amplification of the CYP24 locus on chromosome 20q13.2 [342].

In contrast to degradation of 1,25-$(OH)_2D_3$ by 24-hydroxylase in cancer cells, recently it has become clear that tumor cells contain 1α-hydroxylase activity and thereby are able to generate 1,25-$(OH)_2D_3$. Expression of 1α-hydroxylase has been demonstrated in colorectal cancer [343–345]. It was postulated that in early stages tumor cells respond by up-regulating 1α-hydroxylase activity to counteract neoplastic growth while at later stages of tumor development this is lost [343]. Also in prostate cancer [346] and inflammatory myofibroblastic tumor [347] 1α-hydroxylase has been detected, albeit in the latter case the tumor contains large numbers of macrophages. It can be anticipated that in the coming years investigation of the expression of both 24-hydroxylase, 1α-hydroxylase in tumors will add to the understanding of vitamin D in the initiation and progression of cancer.

VI. STIMULATION OF PROLIFERATION

Over the years a limited number of studies have demonstrated that, in contrast to growth inhibition, 1,25-$(OH)_2D_3$ can also stimulate tumor cell growth and tumor development. In several cells 1,25-$(OH)_2D_3$ has been reported to have a biphasic effect, that is, at lower concentrations ($<10^{-9}$ M) it stimulates proliferation and at higher concentrations (10^{-9} to 10^{-7} M) it inhibits proliferation. However, clear growth stimulation can sometimes be observed not only at low concentrations but also at the concentrations generally found to inhibit tumor cell proliferation and tumor development. 1,25-$(OH)_2D_3$ has been shown to stimulate the growth of a human medullary thyroid carcinoma cell line [348]. Not only cancer cells but also several normal cells, for example, human monocytes [349], smooth muscle

cells [350], and alveolar type II cells [351], are stimulated to grow by 1,25-$(OH)_2D_3$.

Skin is another organ in which different effects of 1,25-$(OH)_2D_3$ have been observed. *In vivo* studies demonstrated that 1,25-$(OH)_2D_3$ and analogs stimulate keratinocyte proliferation in normal mice [352–355] and enhance anchorage-independent growth of preneoplastic epidermal cells [356]. In contrast, other studies showed 1,25-$(OH)_2D_3$ inhibition of proliferation of mouse and human keratinocytes [357,358], and 1,25-$(OH)_2D_3$ is also effective in the treatment of the hyperproliferative disorder psoriasis [359]. Moreover, *in vivo* studies demonstrated that, depending on the carcinogen, 1,25-$(OH)_2D_3$ can either reduce [88] or enhance the induction and development of skin tumors in mice [360,361]. In addition, 1,25-$(OH)_2D_3$ enhances the chemically-induced transformation of BALB 3T3 cells and hamster embryo cells [362,363]. 1,25-$(OH)_2D_3$ also enhanced 12-O-tetradecanoylphorbol-13-acetate-induced tumorigenic transformation of mouse epidermal JB6 Cl41.5a cells [364,365].

Another example comes from research on osteosarcoma cells. In 1986 it was shown that 1,25-$(OH)_2D_3$ stimulated the growth of tumors in athymic mice inoculated with the ROS 17/2.8 osteosarcoma cell line [366]. Earlier the same group reported growth stimulation *in vitro* of these osteosarcoma cells at low concentrations of 1,25$(OH)_2D_3$, but growth inhibition by 10^{-8} M [317]. They speculated that this discrepancy resulted from limited *in vivo* availability of 1,25-$(OH)_2D_3$ for the tumor cells, resulting in concentrations shown to be growth stimulatory *in vitro*. However, in other experiments with nude mice, the availability of 1,25-$(OH)_2D_3$ did not seem to be a factor, as growth inhibition was observed (see Table II). In particular, in nude mice implanted with human osteosarcoma cells (MG-63), growth inhibition and tumor suppression by 1,25-$(OH)_2D_3$ were observed [98]. In two different *in vitro* studies, growth inhibition of MG-63 and growth stimulation of ROS 17/2.8 cells was reported [367,368]. For smooth muscle cells, it has been demonstrated, for example, that growth inhibition or stimulation can depend on the presence of additional growth factors in the culture medium [350]. We followed up on this concept by comparing the effects of 1,25-$(OH)_2D_3$ and analogs on the growth and osteoblastic characteristics of the two osteosarcoma cell lines under identical culture conditions. At concentrations 10^{-10} to 10^{-7} *M*, 1,25-$(OH)_2D_3$ caused an increase in cell proliferation by 100% in ROS 17/2.8 cells, whereas the proliferation of MG-63 cells was inhibited (Fig. 2) [197]. In contrast, in both cell lines 1,25$(OH)_2D_3$ stimulated osteoblastic differentiation characteristics such as production of osteocalcin and alkaline phosphatase activity [197,367]. Analyses with another steroid hormone demonstrated that glucocorticoids inhibited the growth of both osteosarcoma cell lines [369,370]. These data indicate specific differences between these cell lines, especially with respect to the 1,25-$(OH)_2D_3$ growth regulatory mechanisms.

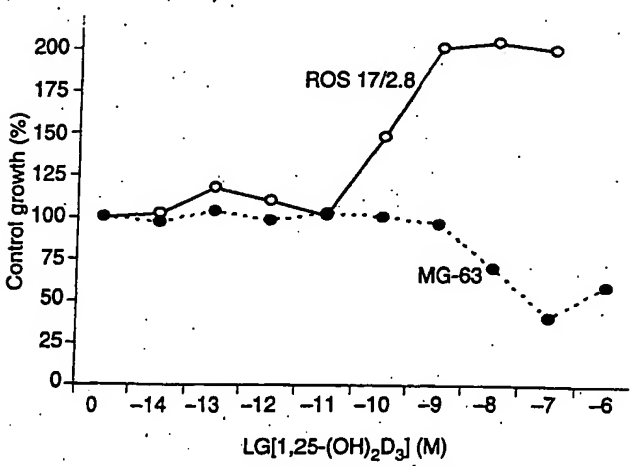


FIGURE 2 Effect of 1,25-$(OH)_2D_3$ on proliferation of the osteosarcoma cell lines ROS 17/2.8 and MG-63. Effects on proliferation were examined as described by van den Bemd *et al.* [197].

Taken together, the data on growth stimulation and tumor development, although detected in only a minority of cancer cells, demonstrate that treatment with 1,25-$(OH)_2D_3$ or analogs may not always cause growth inhibition and tumor size reduction. It is therefore of utmost importance to identify the mechanism(s) by which 1,25-$(OH)_2D_3$ exerts its inhibitory and stimulatory effects on cell growth. This may provide tools to assess whether treatment of a particular tumor will be beneficial. Moreover, purely from a mechanistic point of view, the presence of growth-stimulated and growth-inhibited cells, like the 1,25-$(OH)_2D_3$ sensitive and resistant cells, may provide tools to examine the 1,25-$(OH)_2D_3$ mechanism of growth regulation.

VII. CONCLUSIONS

The data obtained so far, on (1) the distribution of the VDR in a broad range of tumors and (2) the inhibition of cancer cell growth, angiogenesis, metastasis, and PTHrP synthesis by 1,25-$(OH)_2D_3$, all hold promise for the development of treatment strategies based on vitamin D_3 use in a wide range of cancers. Moreover, combination of vitamin D compounds with other antitumor drugs, hormones, or growth factors is an important additional therapeutic option. Throughout the last years data have accumulated on the cellular targets and mechanism of action of 1,25-$(OH)_2D_3$–induced cancer

growth inhibition. The clinical application is enhanced by the development of 1,25-$(OH)_2D_3$ analogs with potent growth inhibitory actions and reduced hypercalcemic activity. At the moment more clinical studies are needed in order to firmly establish whether 1,25$(OH)_2D_3$ and especially vitamin D_3 analogs have therapeutic potential. In the meantime it is crucial to further our understanding of the mechanism(s) by which 1,25$(OH)_2D_3$ exerts its effects on tumor cell growth so that these drugs may be employed more effectively.

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